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Announcement

Medical Research Fellowships

The Division of Medical Sciences of the National Academy of Sciences—National Research Council will accept applications for Postdoctoral Research Fellowships for 1957–58 until December 1, 1956. The Fellowships are awarded in the early Spring. Complete details and application blanks may be obtained from the Division of Medical Sciences, Room 310, National Academy of Sciences—National Research Council, 2101 Constitution Avenue, N. W., Washington 25, D. C.

The Control of Certain Factors of Dosage in Epidermal Carcinogenesis [1]

J. A. McCarter,[2] *Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia*

This communication describes techniques devised for the control of the area and the duration of contact of a carcinogenic agent with the skin of the mouse.

The possibility that the area of skin exposed to the carcinogenic agent may be a factor in determining tumor incidence has been considered by Cramer and Stowell (*1*) and by Calcutt and Powell (*2*). The latter investigators devised a stencil for control of the spread of a solution of benzo-[*a*]pyrene in acetone. The authors reported, however, that a solution placed within the stencil was not confined completely to the area of skin encompassed by the device.

Heidelberger and Weiss (*3*) have reported a technique for confining the carcinogen to a particular area of the skin and for preventing mechanical removal of the agent by rubbing and licking. This technique involved cementing a short length of plastic tubing to the skin of the animal with collodion. Although, in our experience, this technique is useful in certain circumstances (*4*), the injury to the skin caused by the attempts of the animal to dislodge the device led us to abandon it.

The problem of placing an accurately known amount of carcinogen on a certain area of skin is difficult, because any device that is used to contain the solution of the agent "holds up" a portion of the solution. There is a way around this difficulty. Provided that the skin is uniformly wetted or covered by an excess of the solution, the dose of carcinogen received by a given area of skin in a given time should be dependent upon the concentration of carcinogen in the solution and not upon the quantity of the solution applied. The method of exposure adopted in this work has made it unnecessary to know the amount of carcinogen applied. We provide an excess of the agent spread uniformly over a defined area. At the end of a predetermined time interval, during which the mouse is immobilized, the carcinogenic agent remaining on the skin is removed. It is to be expected that, employing the technique described in this paper, the tumor incidence resulting from the exposure of a given area of skin to a

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[2] The author is indebted to the National Cancer Institute of Canada for a grant. He also wishes to express his gratitude to Dr. F. M. Berger, of the Wallace Laboratories, New Brunswick, N. J., for a generous gift of Miltown.

solution of carcinogen would depend upon the concentration of the carcinogenic solution and upon the duration of exposure of the skin to it. That these expectations are fulfilled will be made evident in a subsequent communication (*5*).

Immobilization of Animals

In the experiments that we have done we have immobilized mice for periods of 6 hours' duration or less. In our first attempts to do this, various barbiturates were used singly or combined. The use of these anesthetics produced a high mortality in animals kept for longer periods of time. The advent of the new interneuronal blocking agent "Miltown" (*6*) has made it possible to immobilize mice for periods of 6 hours with negligible mortality. A single intraperitoneal injection of 450 to 480 mg. per kg. body weight produces a flaccid paralysis lasting at least 4 hours. A second injection of one half the original dose, given at the end of 4 hours, extends the duration of paralysis beyond 6 hours. The induction of the paralysis takes place without hyperexcitability. During paralysis the animals lie quietly. Their respiratory rate is not appreciably slowed. Recovery takes place spontaneously without aftereffects and is not accompanied by excitation. The absence of twitching and running movements in the paralyzed animals greatly improves the ease and accuracy with which the technique described in this paper may be used.

A solution of Miltown, 0.9 percent weight per volume, in 5 percent aqueous gum acacia, weight per volume, was used in these experiments.

Technique Employing Nonvolatile Solvents

The hair of the back of the mouse is removed with electric clippers. A circle of filter paper (Whatman #2) cut with a cork-borer is wetted with the carcinogenic solution and any excess of solution is removed with blotting paper. A few trials will acquaint the investigator with the correct amount of solution to use. The circle is then applied to the skin of the immobilized mouse and is held in place with a strip of plastic adhesive tape. It is convenient to use a piece of tape that is long enough to fix the animal to a board or tray. We use a shallow enamel tray covered with a piece of paper toweling. The mice are arranged side by side on the tray which is kept in the dark in an incubator at 25° C. until it is required.

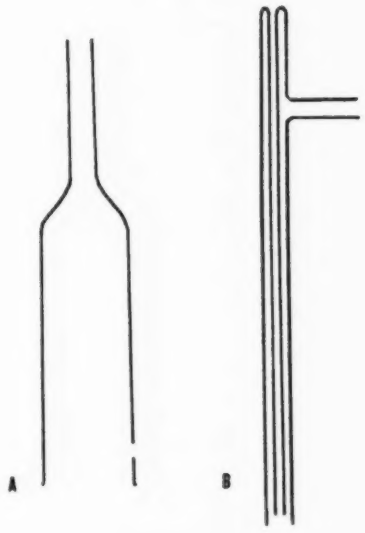
Technique Employing Volatile Solvents

A piece of glass tubing of appropriate diameter and approximately 10 cm. long is cut squarely across at one end. This end is then fire-polished. The other end of the tube is heated and is drawn down to a diameter that will permit attachment to a piece of rubber tubing. A small hole is then blown in the side of the glass tube near the large end (hereafter called the mouth of the device). The diameter of this hole should be just large enough to admit a #25 hypodermic needle.

The device is clamped in an upright position with the mouth downward and the hole in the side at the right (if the operator is right-handed). The

rubber tube is connected to a mercury manometer and to suction. The pressure in the apparatus with the mouth closed is adjusted to approximately 4 cm. of mercury less than atmospheric pressure. The device is illustrated in text-figure 1A.

The hair is removed from the back of the mouse with electric clippers. The immobilized mouse is then picked up in the hand and a fold of the skin is rolled between the thumb and forefinger, so that the skin is held taut over the ball of the forefinger. The skin supported by the forefinger is then pressed lightly against the mouth of the device. Employing a hypodermic syringe fitted with a #25 needle, approximately 0.1 ml. of the carcinogenic solution is injected through the side opening onto the area of



TEXT-FIGURE 1

A. Device for the control of area of spread of solutions in volatile solvents.
B. The washing apparatus.

skin encompassed by the device. The solvent evaporates very quickly, leaving a uniformly covered, circular area of dosed skin corresponding closely in diameter to the outer dimension of the glass tube. The mouse is then fixed to a tray kept in the dark in an incubator at 25° C. until required.

Employing one such device (O. D. 14 mm.) the area of skin dosed with a solution of benzo[*a*]pyrene in acetone was found by measurement to be 1.44 cm.^{2}, with a standard deviation of 0.18 cm.^{2}, in 22 separate measurements. A similar, but larger device (O. D. 18.5 mm.) was found to dose an area of 2.79 cm.^{2}, with a standard deviation of 0.30 cm.^{2}, in 19 separate measurements.

The Washing Procedure

The apparatus used for this procedure is constructed in the following way: A glass tube 15 cm. in length and approximately 4 mm. in diameter is fitted with a short side arm, approximately 2 cm. from one end. A second piece of glass tubing about 2 mm. shorter than the first (and of smaller diameter) is placed within the larger tube and is sealed to it at the end bearing the side arm. The inner tube is left open at both ends. The side arm is connected by means of rubber tubing to a beaker of diethyl ether. The inner tube is connected in the same manner to the water pump. The device is illustrated in text-figure 1B.

The apparatus is held in the hand as if it were a pencil. When the open mouth of the apparatus is pressed against the skin of the mouse a flow of ether is immediately directed onto the skin enclosed by the outer tube and passes up the inner tube to the pump. When the apparatus is lifted from the skin the flow of solvent immediately ceases.

With the aid of this apparatus it is possible to remove the hydrocarbon from the surface of the skin without spreading it over an area larger than that over which it was applied. We employ brief illumination with an ultraviolet lamp (with peak intensity at 3660 Å) to follow the course of the washing procedure. The time required to wash an area of approximately 2 $cm.^2$ is about 2 minutes. We find it convenient to dose the animals employing a schedule of 3-minute intervals.

In order to obtain a measure of the completeness with which the excess of hydrocarbon could be removed from the site, 6 immobilized mice were dosed with a solution of benzo[*a*]pyrene in acetone (0.25% weight per volume) spread uniformly over an area of 2.79 ± 0.30 $cm.^2$ (S.D.). The volume of the solution of carcinogen delivered to the skin was not controlled but was between 0.1 and 0.3 ml.; that is, the amount of carcinogen placed on the skin varied from approximately 250 to 750 μg. The mice were kept in the incubator at a temperature of 25° C.

Exactly 30 minutes after the carcinogen had been applied, the excess of the hydrocarbon remaining at the site was washed off as described above. The dosed circle of skin, together with a wide margin around it, was immediately removed from the animal and the amount of "free" benzo[*a*]-pyrene in the excised skin was determined by a modification of the method described by Miller and Baumann (*7*). The amount of benzo[*a*]pyrene found was 0.56 ± 0.09 (S.D.—6 determinations) μg. per dosed area. It is apparent that the variation found in these analyses was small compared with that in the amounts of benzo[*a*]pyrene applied (250 to 750 μg.). Evidently, the techniques of dosing and washing employed in this experiment may be used to deliver to the skin a reasonably constant dose of benzo[*a*]pyrene.

In a comparable experiment, involving a period of exposure for 5 mice of exactly 60 minutes, the amount of benzo[*a*]pyrene found was 1.04 ± 0.19 (S.D.—5 determinations) μg. per dosed area. The variation in these analyses was also very much smaller than that in the amounts of

benzo[*a*]pyrene applied. The fact that the quantity of benzo[*a*]pyrene found by analysis after 60 minutes' exposure was larger than that found after 30 minutes' exposure under comparable conditions suggested that in the longer period a greater amount of the hydrocarbon had penetrated the skin.

Histologic evidence kindly communicated to the author by Dr. Nicholas Nemethy, of the Department of Anatomy, Dalhousie University, indicates that, at least for short exposures, what is left is in the hair follicles and sebaceous glands. This observation is in agreement with the findings of Graffi (*8*), Simpson and Cramer (*9,10*), and Norden (*11*).

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The Influence of Area of Skin Exposed, Duration of Exposure, and Concentration in Determining Tumor Yield in the Skin of the Mouse after a Single Application of a Carcinogenic Hydrocarbon [1]

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In a discussion of quantitative aspects of epidermal chemical carcinogenesis (*1*) it was pointed out that the number of tumors arising in skin treated topically with a solution of a carcinogenic agent might be expected to depend upon the concentration of the solution, the area of skin exposed, and the duration of exposure to the solution.

That the tumor incidence is dependent upon the area of skin exposed to the carcinogen was indicated by the experiments of Cramer and Stowell (*2*). These investigators used multiple applications of 3-methylcholanthrene in benzene to "large-sized" areas of the backs of Swiss mice and compared the results with those obtained in a similar group of mice painted on a "small" area of the back. A much greater yield of tumor-bearing mice was obtained in the group painted over the larger areas. In spite of this finding, the area of skin exposed to the carcinogen seems to have been neglected as a variable likely to affect tumor incidence in studies of epidermal carcinogenesis. We undertook to examine the effect upon tumor yield of increasing the area of exposure to a single application of a carcinogenic hydrocarbon.

Many previous investigations have been concerned with the question of a possible relationship between tumor incidence and the persistence of a carcinogenic agent for days or weeks at the site of injection or application [reviewed by Norden (*3*)]. To our knowledge there has been no previous investigation of the effects of exposure of only a few hours' duration after a single application of a carcinogenic hydrocarbon to mouse skin. That a short period of exposure might be effective in initiating the production of tumors was suggested to us by Calcutt and Powell's observation (*4*) that mice very rapidly licked off benzo[*a*]pyrene applied to the skin.

It is also of interest that Iversen and Arley (*5*) and Wollman (*6*), in their theoretical consideration of dose-response relationships in carcinogenesis, have considered that the duration of cell exposure to the agent is probably a factor in determining tumor yield. Employing methods

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[2] The authors wish to acknowledge with gratitude that this work was supported by a grant made to one of us (J. A. M.) from the National Cancer Institute of Canada.

devised for the control of duration of exposure (*7*), we undertook to test the influence of this factor on tumor yield.

The effect of the concentration of carcinogen upon tumor yield was studied by Berenblum and Shubik (*8*), who concluded that the tumor yield bears approximately a direct 1 : 1 relationship to the concentration of 9,10-dimethyl-1,2-benzanthracene (DMBA) in 0.06 to 1.5 percent solution in paraffin oil. Their data show a linear relationship between tumor incidence and logarithm of the concentration at least as well as they do a direct arithmetic relationship. Because their data do not provide information about the variation encountered, it is not possible to decide which of these relationships is the more probable. Engelbreth-Holm and Iversen (*9*) found that there was a linear relationship between logarithm (100 minus % tumor incidence) and concentration for a single application of DMBA (0.1 to 1.5% solutions in benzene) to the skin of mice. In the investigation reported in this paper we sought to learn the nature of the dependence of tumor yield on the concentration of DMBA applied once to mouse skin under conditions of known area and duration of exposure.

Material and Methods

Experiment 1. Concentration and Duration of Exposure

Strain CFW [3] female mice, 6 to 10 weeks of age, were used in this experiment. The mice were housed in groups of 15 in plastic, metal-topped cages containing a bedding of sawdust. Water and Purina Fox Chow Checkers were freely available. The cages were kept in a well-ventilated room at a temperature of approximately 25° C. At no time were the mice exposed to bright light. The experiment was begun in June and completed in November 1955.

The plan of the experiment (*see* table 5) involved testing the effects of 3 different exposure times (1, 3, or 6 hours) at each of 3 different concentrations (0.25, 0.50, or 1.0 %) of DMBA [4] dissolved in paraffin oil.[5] The 9 groups of animals each consisted, initially, of 30 mice selected at random. The mice were immobilized with an intraperitoneal injection of Miltown—0.450 gm. per kg. body weight, 0.9 percent weight per volume, in aqueous gum acacia, 5 percent weight per volume. The groups were treated in random order, using the filter-paper-disc method for application of the agent to the midportion of the back (*7*). The diameter of the dosed circle was 16 mm. The excess solution was removed at the end of the appropriate time interval with diethyl ether (*7*). A 10th group of 15 mice was exposed to paraffin oil alone for 6 hours.

Two weeks after the day on which the last animals were treated with carcinogen, each mouse was painted twice weekly with a 2.5 percent solution of croton oil [6] in paraffin oil. This solution was applied to the

[3] Obtained from Carworth Farms, New City, N. Y.
[4] 9,10-Dimethyl-1,2-benzanthracene obtained from Eastman Kodak Co., Rochester, N. Y.
[5] Viscosity 125/135, N. F., obtained from Fisher Scientific Co., Montreal, Que.
[6] Obtained from Fisher Scientific Co., Montreal, Que.

skin with an artist's #6 camel's hair brush, and was spread over an area widely overlapping the dosed area. The efforts of the animal to remove the oil usually resulted in spreading it over most of the skin and fur.

As tumors began to appear, their number, position, and size were recorded in a sketch once each week.[7] Owing to the necessity of avoiding interference with their development, it was not possible to obtain histologic evidence of the nature of the warts. Any wart larger than 2 mm. in diameter (approximately) and observed on 2 consecutive weeks was recorded as a papilloma. The cumulative total of papillomas recorded for each of the 10 groups was plotted weekly in order to learn when a maximum response had been obtained. The experiment was terminated when the croton-oil treatment had been continued for 20 weeks. The data are recorded in tables 1, 2, and 3.

Experiment 2. Area

In this experiment 50 male and 50 female strain I mice were used.[8] The mice were housed and cared for as described in experiment 1.

The mice were divided into 2 groups with equal numbers of male and female animals in each group. The mice of each group were dosed with a solution of benzo[*a*]pyrene,[9] 0.25 percent weight per volume, in acetone (reagent grade). Employing the technique described by McCarter (*7*) for applying volatile solvents, 1 of the groups was dosed with the solution over an area 1.44 ± 0.18 cm.^{2} situated in the mid-portion of the back. The other group was dosed in the same manner and the same region over an area 2.79 ± 0.30 cm.^{2} The excess hydrocarbon remaining at the dosed sites was removed from the back of each mouse by washing with diethyl ether 4 hours after application of the agent to the skin (*7*).

Two weeks after treatment with carcinogen each mouse was painted twice weekly with 2.5 percent croton oil in paraffin oil. The tumor yield was recorded in the manner described in experiment 1. Papillomas began to appear 6 weeks after the croton-oil treatment was started and had almost ceased to appear at the end of the 12th week. In the next 6-week period only 6 new papillomas were recorded. During this time 26 of the female mice died from infection. Our calculations were based on the number of papillomas produced in 12 weeks in the animals that were still alive. The data of this experiment are recorded in table 4.

Analyses of the Data

Experiment 1. Concentration and Duration of Exposure

Incidence of tumors.—The data of the 9 experimental groups were arranged according to the plan of table 5. The horizontal groups were

[7] This technique has been described by Shubik, Baserga, and Ritchie (*10*). Its use helps to overcome the dangers [pointed out by Cramer and Stowell (*2*)] of counting irrelevant swellings and lumps in the skin as papillomas.

[8] The mice were obtained from our own inbred colony which was derived from animals sent us by Dr. H. B. Andervont.

[9] Obtained from Eastman Kodak Co., Rochester, N. Y.

TABLE 1.—*Distribution of papillomas**

Concentration of DMBA (*percent*)	Duration of exposure (*hours*)	No. of mice surviving	Number of mice bearing the following number of papillomas:																	Total no. of papillomas
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
0 (Solvent)	6	15	14	1																1
0.25	1	26	11	7	5	1			2											32
	3	30	17	5	4	3										1				35
	6	25	6	1	4	6	2	2	3		1									71
0.50	1	28	7	2	5	4	3	3	2		2									79
	3	30	13	4	1	5	3	2			1								1	67
	6	25	5	2	3	3	4	3	1		1		1		2					94
1.0	1	27	6	4	4	3	6	2		1	1									70
	3	29	7	2	4	1	3	1	4	1	2	1	1		1			1		123
	6	29	1	6	2	2	1	4	3	3	1	5	1							142

*A circle of skin, 16 mm. in diameter, on the back of each mouse was exposed for the periods of time noted in the table. After a 2-week interval, all mice were painted twice weekly for 20 weeks with a 2.5 percent solution of croton oil in paraffin oil.

TABLE 2.—*Latent period of all papillomas observed**

Concentration (*percent*)	Exposure (*hours*)	Number new papillomas observed in following number weeks:														Total
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	
0. 25	1			17	7	1	4			1				2		32
	3		1	20	3	3	1	3	1		2	1				35
	6			5	36	9	10	5	2	2	2					71
0. 50	1		2	7	32	2	20	7	5	1	3					79
	3		11	29	9	4	7	1		4	1	1				67
	6	6	11	9	22	14	9	7	5	1	1	5	3	1		94
1. 0	1		3	7	11	5	13	15	3	2	7		2	2		70
	3		6	41	22	13	14	16	5	2	2			2		123
	6		3	37	43	17	20	9	4	3	2	1	1	2		142
Total		6	37	172	185	68	98	63	25	16	20	8	6	9	0	713

*The numbers in the columns are those of the new papillomas observed in a particular week.

TABLE 3.—*Latent period of first papilloma which appeared in a mouse**

Concentration (*percent*)	Exposure (*hours*)	Number mice bearing first papilloma in following number weeks:														Total
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	
0. 25	1			7	4		2			1				1		15
	3		1	7	2	1	1				1					13
	6			4	9	2	1	2	1							19
0. 50	1		1	2	10	1	3	2	1		1					21
	3		5	10	1		1									17
	6	2	2	4	7	3	1	1								20
1. 0	1		2	3	6	2	2	5	1							21
	3		1	12	6			1				1				22
	6		3	8	8	4	4							1		28
Total		2	15	57	53	13	15	11	3	2	2	1	0	2	0	176

*The numbers in the columns refer to the numbers of tumor-bearing mice newly recorded in a particular week.

TABLE 4.—*Distribution of papillomas in mice, according to area of exposure and sex, after a single application of benzo[a]pyrene in acetone**

Area of exposure	Total mice	Total papillomas	No. mice bearing following no. papillomas:			
			0	1	2	3
		Males				
1.44 cm.² S.D.=0.18	23	8	15	8	—	—
2.79 cm.² S.D.=0.30	23	14	12	9	1	1
		Females				
1.44 cm.² S.D.=0.18	23	8	16	6	1	—
2.79 cm.² S.D.=0.30	19	17	9	3	7	—

*Duration of exposure was 4 hours. After an interval of 2 weeks all mice were painted twice weekly with 2.5 percent croton oil in paraffin oil.

TABLE 5.—*Experimental plan*

Duration of exposure (*hours*)	Concentration (*percent*)		
	0.25	0.50	1.0
1	A	B	C
3	D	E	F
6	G	H	I

TABLE 6.—*Yield of papillomas in mice treated once with different concentrations of DMBA in paraffin oil for different lengths of time**

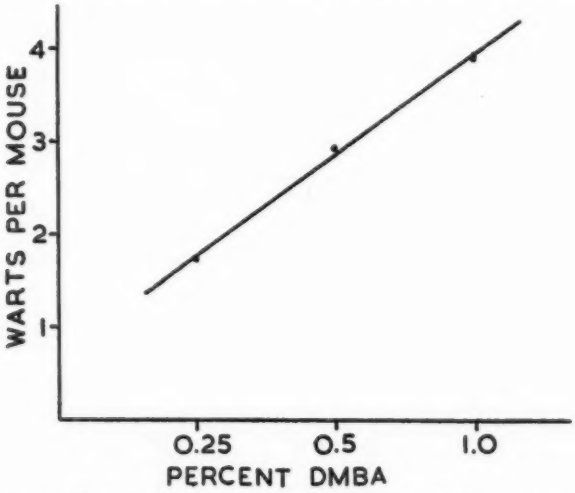
Concentration of DMBA (*percent*)	Yield of papillomas in relation to duration of exposure (*hours*)			Average
	1	3	6	
0. 25	1. 23	1. 17	2. 84	1. 75
0. 50	2. 82	2. 23	3. 76	2. 94
1. 0	2. 59	4. 24	4. 90	3. 91
Average	2. 21	2. 55	3. 83	

*Calculated from the data of table 1.

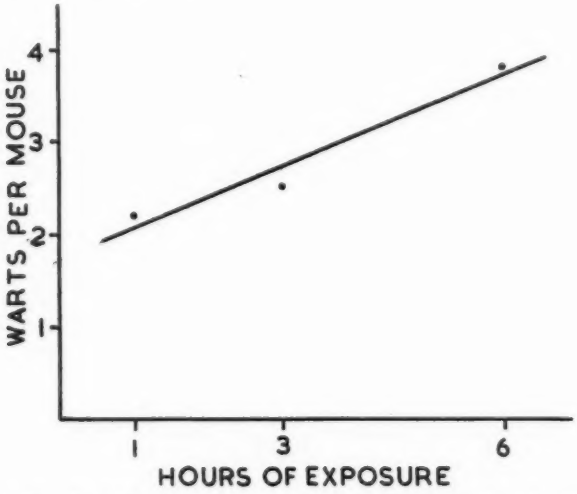
exposed for an identical time; the vertical groups were exposed to identical concentrations of the agent.

An analysis of variance of the data in table 1 was done according to Snedecor (*11*) in order to assess the effects of the logarithm of the concentration and the duration of exposure on the tumor yield. The data were not corrected for the control tumor incidence (1 papilloma in 15 mice). Wollman (*6*) has drawn attention to the uncertainty of how to correct for control incidence. The average number of papillomas produced in each of the 9 groups of treated animals was calculated, and these averages appear in table 6.

The analysis of variance showed that there was a very highly significant variation in tumor incidence due to the different log concentrations ($P<0.001$), and there was no evidence that the regression line fitted by the least-squares method was not straight ($P>0.2$). *See* text-figure 1.



TEXT-FIGURE 1.—Variation in tumor incidence due to different concentrations of DMBA. The line corresponds to the theoretical equation $Y=3.947+3.595$ log percent DMBA. Standard error of the slope=0.709. The data of the last column of table 6 provided the points.



TEXT-FIGURE 2.—Variation in tumor incidence due to duration of exposure. The line corresponds to the theoretical equation $Y=1.758+0.332$ (hours of exposure). Standard error of the slope=0.0848. The data in the last line of table 6 provided the points.

TABLE 7.—*Latent periods (in weeks) for appearance of all papillomas**

Concentration of DMBA† (*percent*)	Latent period in relation to duration of exposure‡ (*hours*)			Average
	1	3	6	
0. 25	10. 47	10. 43	10. 97	10. 62
0. 50	11. 20	10. 04	11. 10	10. 78
1. 0	12. 30	10. 78	10. 82	11. 30
Average	11. 32	10. 42	10. 96	

*Calculated from data of table 2.

†The regression equation for latent period on concentration was $Y = 11.09 + 0.704$ (log percent DMBA). The standard error of the slope was 0.369.

‡The regression equation for latent period on duration of exposure was $Y = 11.19 - 0.0665$ (hours of exposure). The standard error of the slope was 0.0408.

The analysis of variance also showed that there was a very highly significant regression of tumor yield as the duration of exposure was increased ($P<0.001$). There was no reason to suppose that the regression line was not straight ($P>0.2$). *See* text-figure 2.

There was no evidence of interaction between concentration and time of exposure in their effect on tumor yield.

Latent period of all papillomas.—The data of table 2, showing the time at which each papilloma appeared, were arranged according to the plan of table 5. The average latent periods were calculated for each of the 9 groups and are shown in table 7.

Examination of these averages (*see* last column of table 7) suggested that the latent period for all the papillomas was lengthened as the concentration of carcinogen was increased. Statistical analysis revealed that there was an increase in latent period dependent upon log concentration ($P\sim0.05$) but not upon time (*see* table 7 for regression equations). We thought it possible that the average rate of development of a large number of papillomas in the treated area might be slower than that of a small number—an effect due, perhaps, to a limitation in the supply of nutrient materials to the growing papillomas. An analysis of the data, conducted according to Snedecor (*11*), showed that there was no correlation between the number of papillomas produced in a mouse and their average latent period. The significance of these findings is discussed later in this paper.

Latent period of first papillomas.—The same statistical analytical procedure was applied to the data of table 3, in which are indicated the times at which each papilloma-bearing mouse was first recorded. The mean latent periods are given in table 8. The analysis provided no evidence that the mean latent period for the production of the first papilloma recorded is dependent upon duration of exposure or log concentration of carcinogen. *See* table 8 for regression equations.

Experiment 2. Area

Employing the data of table 4 for the distribution of papillomas according to area and sex, the average number of papillomas produced in each

TABLE 8.—*Latent periods for the appearance of the first papillomas**

Concentration of DMBA† (*percent*)	Latent period in relation to duration of exposure‡ (*hours*)			Average
	1	3	6	
0.25	10. 73	10. 00	10. 53	10. 42
0.50	10. 90	8. 94	9. 70	9. 85
1.0	10. 85	10. 05	10. 25	10. 38
Average	10. 83	9. 66	10. 16	

*Calculated from data of table 3.
†Regression equation for latent period on concentration was $Y = 10.25 - 0.0144$ (log percent DMBA). Standard error of the slope = 0.596.
‡Regression equation for latent period on duration of exposure was $Y = 10.61 - 0.107$ (hours of exposure). Standard error of the slope = 0.0685.

experimental group was calculated. These averages are entered in table 9. The data of table 4 were subjected to an analysis of variance in order to learn the effect of area and sex on the tumor yield. The analysis revealed that there was a highly significant dependence of tumor yield upon area of skin exposed to the carcinogenic agent ($P \sim 0.01$). There was no evidence to suggest that the incidence of papillomas in the male mice was different from that in the female mice ($P > 0.2$). There was no interaction of sex and area of exposure affecting tumor incidence. The regression line is plotted in text-figure 3.

TABLE 9.—*Number of papillomas produced in treated area, according to area and sex**

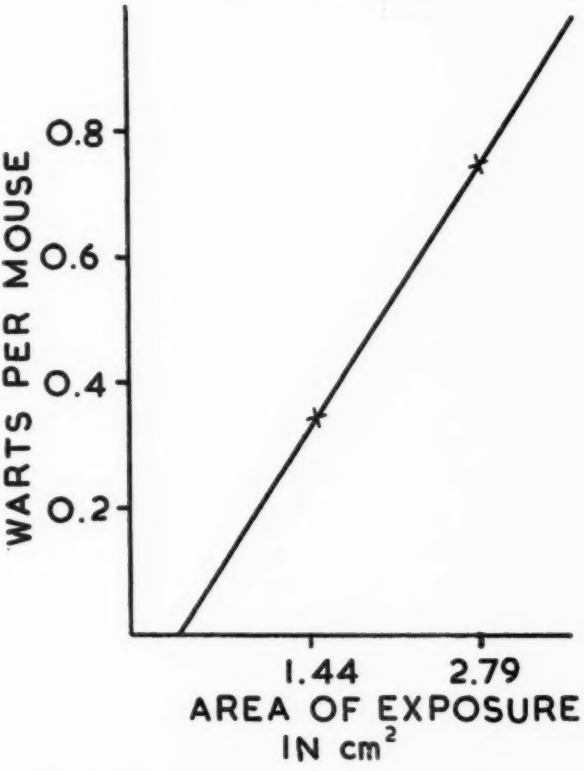
Area	Male	Female	Average
1.44 cm.²	0. 348	0. 348	0. 348
2.79 cm.²	0. 609	0. 895	0. 752
Average	0. 479	0. 622	

*Calculated from the data of table 4.

Discussion

In these experiments tumor yield was measured by recording the number of papillomas produced in each treated animal and also by recording the proportion of treated mice that bore papillomas. Our data indicate that the former index provided more information than the latter and was a more accurate measure of the tumor yield. The first index provides measurement data which permit an analysis of variance and, hence, yield more information than do the enumeration data provided by the second index. Enumeration data cannot be subjected to an analysis of variance.

An additional disadvantage of the second index is evident from a consideration of the data of table 1, which reveal that in every treated group more than 40 percent of the mice bore tumors. The minimum and maximum values were 43 and 96 percent, respectively. Because these data are not balanced by lower responses, the method of probit analysis cannot be accurately applied. Tentative analysis of the tumor-bearer



TEXT-FIGURE 3.—Variation in papilloma incidence due to different areas of exposure. The points were obtained from the marginal averages of table 9. The equation of the line passing through the points is $Y = -0.067 + 0.289$ (area in cm.^{2}). Standard error of the slope = 0.111. The intercept on the Y axis (-0.067 papillomas per mouse for area = 0) did not differ significantly from zero ($P > 0.5$), calculated according to Snedecor (*11*).

data with this method showed that there was a relationship between log concentration and tumor yield but did not reveal that there was a dependence of tumor yield upon duration of exposure. Both of these relationships emerged when the measurement data provided by the first index were subjected to an analysis of variance.

Analysis of variance demonstrated the existence of a relationship between tumor yield and log concentration (*see* text-fig. 1). The analysis

also revealed that this relationship did not deviate significantly from linearity. A dependence of response on log concentration is characteristic of the pharmacologic behavior of many drugs. A dependence of tumor yield upon log dose was demonstrated in mice by Bryan and Shimkin with subcutaneous injections of solutions of carcinogenic hydrocarbons (*12,13*).

Engelbreth-Holm and Iversen (*9*) found a linear relationship between log (100 minus % incidence) and concentration in experiments involving a single application of DMBA to mouse skin. However, the present investigation cannot be said to confirm the findings of Engelbreth-Holm and Iversen. In their investigation mice were dosed once with 0.05 ml. of solutions of DMBA of different concentrations in benzene. It is apparent that, because of rapid evaporation of the solvent, the skins of the mice could not have been exposed to different concentrations of DMBA for more than a few minutes. Rather, they must have been exposed to different *amounts* of DMBA of very nearly the same concentration. This fact was pointed out by Berenblum and Schoental in another connection (*14*). The data of Engelbreth-Holm and Iversen (*loc. cit.*) show that the rate of disappearance of the hydrocarbon from the dosed site is a function of the amount applied to the skin. The question is raised whether their findings can be interpreted as a dependence of tumor yield upon concentration, except as the latter determines the amount of carcinogen applied. Perhaps their data demonstrate a dependence of tumor yield upon persistence of the agent at the site, but, because the duration of exposure was not controlled, it is not possible to assign the observed effects to any particular variable.

So far as we are aware, there has been no previous demonstration of the dependence of tumor yield upon duration of exposure of the skin to a single application of carcinogenic hydrocarbon. The demonstration of the existence of this factor was made possible by our ability to maintain a constant carcinogenic stimulus to the skin and this, in turn, was made possible by the immobilization of the animals. The usual methods of application of carcinogenic agents to mice allow the animals to lick and rub them from their skins (*4*). In this way mice may remove different applied substances at different rates and may, therefore, allow one substance to remain where applied longer than another. Because the tumor yield depends upon the duration of exposure of the skin, the uncontrolled removal of different substances is liable to complicate the comparison of their carcinogenic potencies.

Recently, Graffi, Scharsach, and Heyer (*15*) reported that the oral administration of DMBA to mice caused initiating action in the skin. This finding strongly emphasizes the importance of preventing mice from licking carcinogenic substances from the skin.

The reason for the dependence of tumor yield upon the duration of exposure is not clear. It is not known whether time is a factor because it is required for establishment of a certain effective concentration in the skin, for penetration of a certain amount of the carcinogen, or for

other reasons. It is not known whether dormant tumor cells are produced during the exposure or subsequent to it. We are attempting to answer these questions.

It is of interest that the tumor yield, calculated from the equation of the regression line in text-figure 2, is 1.76 papillomas per mouse for 0 hours of exposure. This figure is very much larger than the measured control incidence, which was 0.066 papillomas per mouse. There is no reason to doubt that the regression line is straight over the period 1 to 6 hours of exposure. Evidently, the line is not straight between 0 and 1 hours.

Possibly the reason for this discrepancy may be found in studies that are being conducted in our laboratory on the rate of accumulation of carcinogenic hydrocarbons in the skin of immobilized mice. These studies indicate that the concentration of hydrocarbon in the epidermis increases during the first hour or two but thereafter increases very slowly or not at all.

Also of interest is that Weigert, Calcutt, and Powell (*16*), in their studies of the rate of formation of BPX_2 in mouse skin, found that the amount of metabolite increased little or not at all during the interval 2½ to 5 hours after the application of benzo[*a*]pyrene to animals fixed so that they could not move (*see* fig. 2 in their paper). The reader is referred to Norden's extensive study of the penetration of benzo[*a*]pyrene into mouse skin (*3*).

The analysis of our data for the latent period of the first papillomas provided no evidence of a dependence of latent period on duration of exposure or on concentration. These data support the theory of the 2-stage mechanism of carcinogenesis advanced by Berenblum and Shubik (*17*).

Our finding that the average latent period of all the papillomas increased with increasing concentration of DMBA (*see* table 7) cannot be explained by supposing that multiple papillomas produced in the treated area of skin influenced each other's development. There was no correlation between the number of papillomas produced and their latent periods. In this experiment, evidently, the papillomas developed independently of each other. Shubik, Baserga, and Ritchie (*10*) have provided descriptions of the independent progression of morphology and growth rate in the life of papillomas in mice. Our data show that the papillomas induced by a single application of the highest concentration of DMBA used in our experiment included a larger proportion of tumors that developed slowly than were included in those papillomas induced by the use of lower concentrations of the carcinogen. The possibility that the character of the tumors varied in a systematic way with the concentration of the carcinogen is being investigated.

The demonstration that tumor incidence varies directly with the area of skin exposed to a carcinogenic hydrocarbon is a confirmation of the earlier finding of Cramer and Stowell (*2*). In our experiments, doubling the area of exposure doubled the yield of papillomas. It is apparent that

the source of much variation in the experimental production of skin tumors can be removed by close control of the area of exposure.

Summary

1) Skin tumors were produced in strain CFW female mice in response to croton-oil treatment following a single application of solutions of 9,10-dimethyl-1,2-benzanthracene in paraffin oil to a constant area of skin. Three different concentrations of the hydrocarbon (0.25, 0.50, and 1.0%) and 3 different exposure times (1, 3, and 6 hours) were employed.

2) Analysis of variance of the number of papillomas produced revealed that the tumor yield was a linear function of the logarithm of the concentration of carcinogenic hydrocarbon.

3) Analysis of variance of the number of papillomas produced revealed that the tumor yield was dependent on the duration of exposure of the skin to the carcinogenic agent. The analysis provided no evidence that the regression line deviated from linearity for exposure times of 1 to 6 hours.

4) Analysis of the data for the latent periods of all papillomas revealed that there was a significant dependence of latent period on concentration but not on duration of exposure. An analysis of variance of data for the latent period of the papilloma appearing first showed that there was no reason to suppose that there was a dependence of latent period on concentration or on duration of exposure.

5) Employing a single application of benzo[*a*]pyrene in acetone to the skin of strain I mice of both sexes it was shown that for constant exposure time the tumor yield was directly proportional to the area of skin exposed. No evidence of a dependence of tumor yield upon the sex of the mouse was obtained.

6) The implications of these findings for the study of epidermal carcinogenesis were discussed. It was pointed out that the persistence of a carcinogenic agent at the site of application to the skin should be controlled in experiments designed to compare the carcinogenic potencies of different substances. It was also pointed out that the variability experienced in measurements of tumor yield could be reduced by control of the area of skin exposed to the carcinogenic agent.

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Effect of Nitromin on Mitosis and Cytoplasmic Volume in the Cells of Two Mouse Ascites Tumors[1]

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The characteristic action of the nitrogen mustards on a wide variety of living systems has been observed by numerous investigators [*e.g.* (*1–4*)]. More recently, a new member of this series, nitrogen mustard *N*-oxide (Nitromin), has been synthesized by Ishidate and his associates at Tokyo University (*5–8*) and is now being studied intensively in Japan, both experimentally and clinically, in the treatment of a variety of human neoplasms.

Tested against the cells of the Yoshida sarcoma (*9*), Nitromin proved to be similar in its cytologic action to the original nitrogen mustards (*10*) but was considerably less toxic (*11*). Thus, the maximum total dose (MTD) for a single intraperitoneal injection of original nitrogen mustard (HN_2) in rats bearing the Yoshida sarcoma was found by Yoshida to be 1 mg. per kg., whereas the MTD for Nitromin was 40 mg. per kg. (*12*). Moreover, repeated doses of 10 to 20 mg. per kg., administered via intraperitoneal, subcutaneous, or oral routes to rats bearing the Yoshida sarcoma, produced prolongation in survival time without development of toxic symptoms (*12*).

An indication of the toxicity of Nitromin in non-tumor-bearing mice is shown in table 1. The MTD for a single intraperitoneal injection in C3H mice was 80 γ per gm., and the LD_{50} about 150 γ per gm.

In the present investigation are described the major qualitative and quantitative changes in the mitotic process which occurred in cells of sarcoma 37 and in an ascites hepatoma of the mouse following administration of Nitromin. Data are presented on the relatively enormous increases in cell volume which result, apparently, from the continued synthesis of cytoplasm in the absence of cell division.

Materials and Methods

The ascites form of sarcoma 37, carried intraperitoneally through 150 transfer generations in CAF_1 hybrid mice, and a recently developed free-

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cell ascites hepatoma 129(P) (*13*), carried intraperitoneally through 90 transfer generations in C3H/HeN mice, were used in this study. Animals of both sexes ranging from 18 to 25 gm. were inoculated intraperitoneally with about 0.05 cc. of 7-day-tumor ascites. Under these conditions, most mice bearing sarcoma 37 ascites die in 10 to 11 days. The range of survival of mice bearing hepatoma 129(P) is 12 to 23 days.

Four or 5 days after inoculation, the mice received, except where otherwise indicated, a standard intraperitoneal injection of 40 or 50 γ per gm. of a freshly prepared solution of Nitromin [5] dissolved in sterile 0.85 percent saline.

The cytologic effects of Nitromin on sarcoma 37 and hepatoma 129(P) were observed to be almost identical. While sarcoma 37 was used chiefly for the quantitative analysis, cells of ascites hepatoma 129(P) were found to be very favorable material for qualitative analysis and were used exclusively in the preparation of photomicrographs.

For cytologic study, samples of the tumor ascites were withdrawn from each of several animals with a sterile glass pipette and then fixed and

TABLE 1.—*Toxicity of Nitromin in non-tumor-bearing C3H mice following a single intraperitoneal injection*

Dose of Nitromin (γ/gm.)	No. of deaths among 10 mice
10	0
20	0
40	0
80	0
100	1
150	5
200	3*

* Total of 7 mice.

stained with an equal or double volume of a 1 percent solution of acetic orcein (dissolved in 50% acetic acid). All measurements and cell counts were made from material prepared in this way. Figures 9 and 10 were obtained from air-dried, Giemsa-stained smears of the ascitic fluid.

For the measurement of cell diameters, a droplet of tumor ascitic fluid, fixed and stained as described above, was placed on a glass slide and covered lightly with a coverslip without squashing. Diameters were measured by means of a filar micrometer eyepiece, and the cell volume (as spheres) in cubic micra was calculated from these values.

In text-figure 1, each point on the experimental curve represents the average of 5 mice. The controls represent the average values for the ascites of 3 mice. The data of text-figures 2, 3, and 5 were obtained, except where otherwise indicated, by repeated withdrawal of ascitic fluid from the same mice at the time intervals indicated. A small part of the data of text-figure 4 was also obtained by repeated withdrawal of ascites

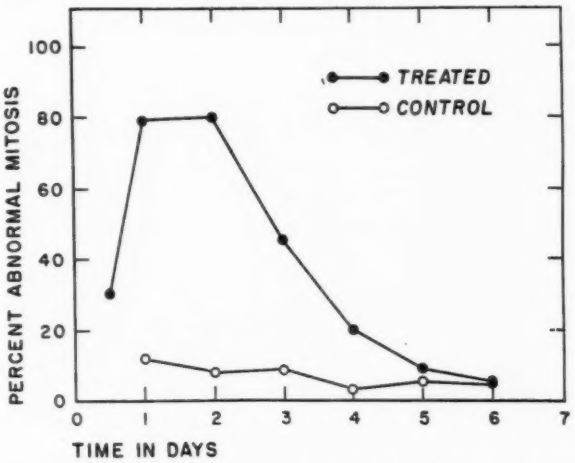
[5] We wish to thank the Takeda Pharmaceutical Industries, Ltd., Osaka, Japan, for their generous supply of Nitromin [methyl-bis(β-chloroethyl)-amine-*N*-oxide HCl].

from the same animals. The remaining values represent samples of ascites obtained at random from other mice of the same group.

Results

The time course for the increase in mitotic abnormalities in sarcoma 37 following a single intraperitoneal injection of 40 γ per gm. of Nitromin is shown in text-figure 1. The untreated cells of sarcoma 37 are characterized by a relatively high number of abnormal mitotic figures. Thus, cells with 1 or several relatively thin chromosome bridges of the type described by Koller (*14*) occur rather frequently among the anaphases of the control, untreated ascites. The chromosome bridges in post-metaphase cells affected by the action of Nitromin, however, usually consisted of several thick strands together with lagging or acentric chromosome fragments and coagulated chromatin elements, which presented a typical, easily recognizable picture. Thus the scoring of abnormal mitoses in the treated tumors represents minimum values and includes only those mitotic figures which clearly indicate the effects of the drug, as shown in figures 1 to 8.

The highest percentage of abnormal mitoses appeared between 24 and 48 hours after injection. This is in agreement with most of the earlier work with the nitrogen mustards and indicates an effect upon some part of interphase which does not become manifest until the cell enters mitosis (*4,15*). It is interesting to note that the maximum values for abnormal mitosis even at 24 to 48 hours never exceeded about 80 percent. Thus, normal mitoses were always observed in treated cells, and the question arises as to whether such cells were actually resistant to the action of Nitromin or simply were not exposed initially to a sufficient concentration of the drug.

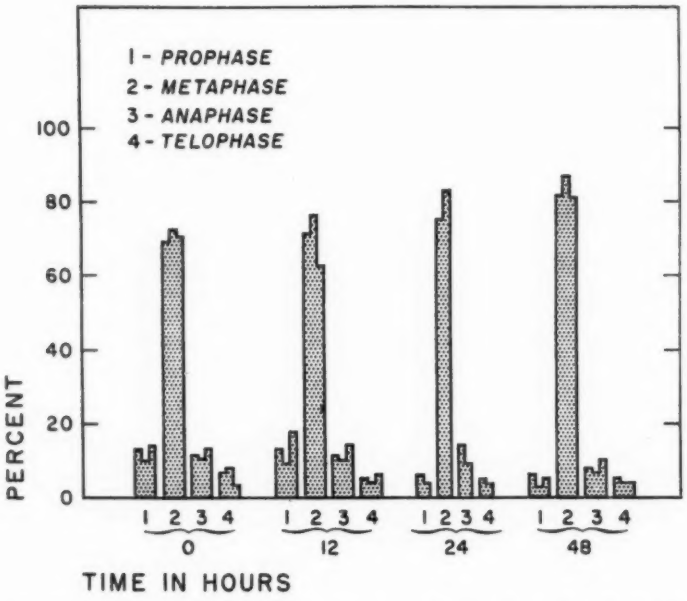


TEXT-FIGURE 1.—Changes in the percentage of abnormal mitoses in sarcoma 37 ascites-tumor cells following a single intraperitoneal injection of 40 γ per gm. of Nitromin. Each point on the treated curve is the average of 5 mice, and 100 mitotic cells were analyzed for each mouse.

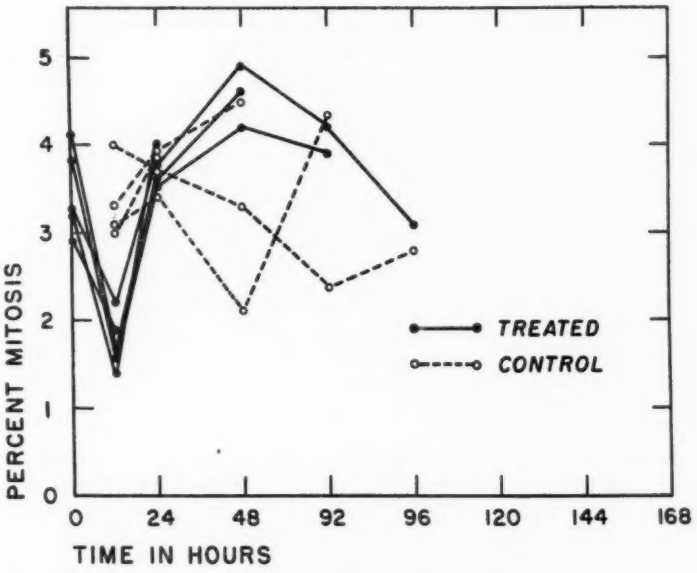
All the major classical cytologic effects of nitrogen mustard were also observed in the present study with Nitromin. Figures 1 to 8 and figures 11 and 12 illustrate some of the main consequences which accompany the action of Nitromin on cells of ascites hepatoma 129(P). Chromosome bridges, consisting of several strands or of a single coagulated strand, acentric fragments, and various degrees of coagulation of chromosomes were frequently observed, particularly in the early hours after injection (figs. 1 to 4). For the most part, the mitotic abnormalities consisted chiefly of metaphase figures containing scattered chromosomes and/or chromosomes in various states of coagulation (figs. 5 to 8). Several days after injection the ascites was marked by the appearance of mononucleate and binucleate giant cells (fig. 11) and by cells in karyorrhexis (fig. 12). The appearance of untreated ascites-tumor cells of hepatoma 129(P) is shown in figures 9 and 10.

The question as to whether the tumor cells shown in the accompanying figures were irreversibly damaged by treatment with Nitromin, while of considerable importance, could not be determined from the qualitative data presented in this study.

The appearance of large numbers of abnormal metaphases gave the impression that these cells were arrested and were accumulating in metaphase in preference to other mitotic phases. That this increase was



TEXT-FIGURE 2.—Histogram showing percentage changes in each mitotic phase in sarcoma 37 ascites-tumor cells following a single intraperitoneal injection of 40 or 50 γ per gm. of Nitromin. Each small peak represents the count from 1 mouse, and these counts are shown in the same relative position at each time interval. A total of 200 mitotic cells was analyzed for each mouse.

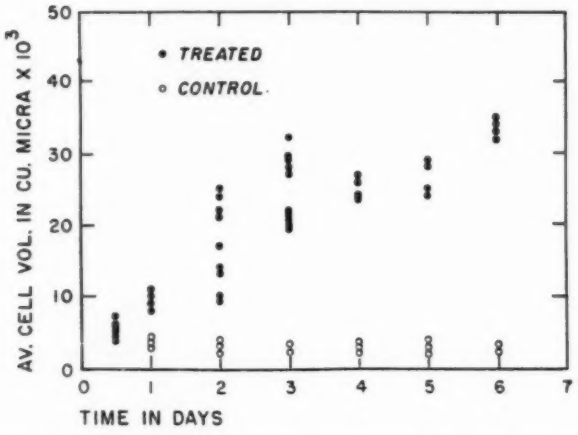


TEXT-FIGURE 3.—Transitory decrease in mitotic rate in sarcoma 37 ascites-tumor cells following a single intraperitoneal injection of 40 or 50 γ per gm. of Nitromin. Each point represents an individual mouse in which 2,000 cells were counted.

actually very slight and did not disturb appreciably the proportion of cells in each mitotic phase may be seen in text-figure 2. Thus, about 60 to 90 percent of all mitotic figures were in metaphase throughout the 48-hour period with only a slight increase in 24 to 48 hours. Prophase values at 24 to 48 hours dropped slightly, but anaphase and telophase levels were relatively constant despite the increase with time in the percentage of all abnormal mitotic cells.

Several workers have observed that one of the earliest effects of the nitrogen mustards is to cause a transitory decrease in the number of cells entering mitosis (*2,3,15,16*). In sarcoma 37 ascites-tumor cells, Nitromin likewise produced a transitory decrease at 12 hours in the mitotic rate, followed by a slight rise in some mice, and a return at 48 hours to normal values (text-fig. 3).

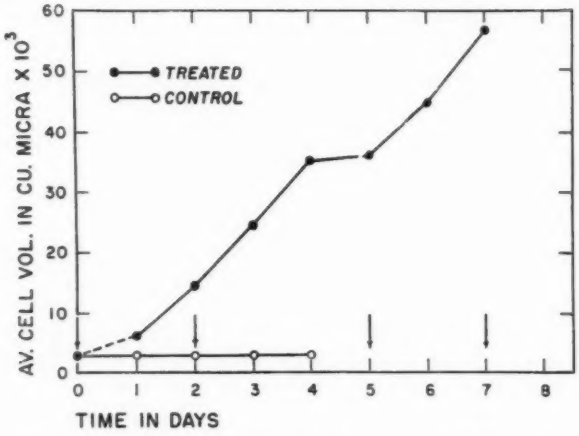
The inhibitory effects on the initiation of cell division and the production of abnormalities in actively proliferating cells have usually been described as the major consequences of the action of nitrogen mustard. There are in addition, however, several references to the formation of "giant" cells which may owe their size to increased growth (synthesis) in the absence of mitosis (*15,17,18*). In tissues such enlarged cells do not constitute the majority of the cell population, but in the case of free-cell suspensions, where the drug is in direct contact with most cells, a more uniform volume increase may be expected to occur. The increase in average cell volume in sarcoma 37, following a single intraperitoneal



TEXT-FIGURE 4.—Changes in cell volume in sarcoma 37 ascites-tumor cells following a single intraperitoneal injection of 40 γ per gm. of Nitromin. Each *solid circle* represents an individual mouse in which 50 to 100 cells were measured.

injection of 40 γ per gm. of Nitromin, is illustrated in text-figure 4. The values vary widely among the cells of a given population as well as from one animal to another, but a slight increase in cell volume can be detected as early as 12 hours post injection. The largest cells measured were attained at 6 days and ranged from 30×10^3 to 40×10^3 cubic micra, which corresponds approximately to an 11- to 12-fold increase in average cell volume over that of the untreated cells.

It should be noted that normal mitoses were almost never observed in cells which showed a large increase in volume. Thus, giant cells were either



TEXT-FIGURE 5.—Changes in cell volume in hepatoma 129(P) ascites-tumor cells following 4 separate injections of 40 γ per gm. each of Nitromin. *Arrows* indicate the days on which Nitromin was administered.

in abnormal mitosis, in normal interphase with an intact nucleus, or showed various degrees of karyorrhexis. Normal mitosis, especially several days after injection, appeared to be almost exclusively a product of cells of nearly normal size, and it is these cells which presumably were responsible for continued growth of the tumor.

The volume increase for cells of the ascites hepatoma following 4 separate injections of Nitromin is shown in text-figure 5. The average cell volume at 7 days was 57×10^3 cubic micra or 19 times the volume of untreated cells. This population, consisting largely of such giant cells, persisted for several days in the ascites without signs of mitosis when transplanted intraperitoneally into new mice. After a latent period of about 2 to 4 weeks, however, cells of normal size, some of which were in normal mitosis, began to appear in the ascites. The fate of the "giant" cells is not clear, but they gradually decreased in number and were soon replaced by large numbers of normal cells.

Discussion

The effects obtained with Nitromin in this study appear to be similar to those observed by Yoshida for the Yoshida sarcoma and to the results obtained in the literature with nitrogen mustard. Thus, Nitromin produced a variety of abnormalities in mitotic cells, caused a transitory decrease in the mitotic rate at 12 hours, but did not cause an arrest or accumulation of cells in any phase of the mitotic process.

The action of Nitromin is twofold, for, in addition to its action against proliferating cells, it actually inhibits some cells from entering division (fig. 3), an inhibition which results in the formation of relatively enormous cells.

At the time Nitromin is administered, cells already in division, as well as those cells which continue to enter division while the drug is still active, show the typical cytologic effects described. Also, it appears that cells in certain stages of interphase may also respond to Nitromin, but the effects do not become manifest until the cells enter division.

In addition, however, there is a stage of interphase in which the cells are particularly sensitive to Nitromin. Cells exposed to Nitromin at this point in their life history, as well as those cells which enter this stage while the drug is active, are prevented for long periods of time from entering the mitotic process. Such cells nevertheless continue to increase in cell volume, apparently as a result of cytoplasmic synthesis in the absence of division. Thus the administration of Nitromin results both in mitotic abnormalities and indirectly, through a blockage of cell division, in the production of "giant" cells.

The appearance of giant cells as a result of administration of Nitromin was also observed in the case of the Yoshida sarcoma and was interpreted as the result of abnormal mitosis which produced polyploidy. Koller and Casarini (*18*), using nitrogen mustard, observed a fourfold increase in the volume of interphase cells in the Walker carcinosarcoma 256 grown

subcutaneously. Such changes were considered by them as characteristic of degenerating cells, but the absence of micronuclei indicated that these alterations could not be attributed to a defective nucleus. Instead, the effects were interpreted as due to a nonspecific metabolic disturbance in the cytoplasm brought about directly by nitrogen mustard.

On the other hand, Klein and Forssberg observed the enlargement of Ehrlich ascites-tumor cells following X irradiation. Their determination of the nitrogen and pentose nucleic acid values, expressed as average amounts per cell, indicates that "real synthesis of new cell substance occurs and not merely a swelling due to water uptake" (*19*). Likewise, the results of Gardella and Lichtler (*20*) on the effect of radiation on the Yoshida sarcoma and those of Friedman *et al.* (*21*) both point to this same general conclusion.

Our interpretation of the volume increase which follows the administration of Nitromin, although not based on quantitative chemical data, is in agreement with that of the above investigators.

Summary

1) The effects of Nitromin [methyl-bis(β-chloroethyl)-amine-*N*-oxide HCl] on sarcoma 37 ascites-tumor cells and on the cells of the mouse ascites hepatoma 129(P) were observed.

2) The cytologic effects on sarcoma 37 following an intraperitoneal injection of Nitromin were almost identical with those observed in the case of the Yoshida sarcoma after treatment with Nitromin, as well as with the classical effects on various types of cells reported in the literature for the nitrogen mustards.

3) Following a single intraperitoneal injection of 40 γ per gm. of Nitromin, the percentage of mitotic abnormalities slowly increased to a maximum of 80 percent at 24 to 48 hours and then gradually returned to normal. A single injection of Nitromin, however, did not alter appreciably the percentage of cells in each mitotic phase.

4) A single intraperitoneal injection of 40 or 50 γ per gm. of Nitromin produced at 12 hours a transitory decrease in the mitotic rate of sarcoma 37 cells, followed by a slight elevation in mitotic rate in some animals, and a return at 48 hours to normal values.

5) Large increases in cell volume were observed in both tumors following administration of Nitromin. In sarcoma 37 ascites-tumor cells, a single intraperitoneal injection of 40 γ per gm. caused a gradual increase in average cell volume which reached a value in 6 days of 32 to 35 $\times$ 10^3 cubic micra, corresponding to an 11- to 12-fold increase in volume over untreated cells. In the cells of hepatoma 129(P), 4 separate injections of 40 γ per gm. each of Nitromin in a 7-day period produced an increase in average cell volume which reached a value in 7 days of 57 $\times$ 10^3 cubic micra, corresponding to a 19-fold increase in volume over untreated cells.

6) The effects of Nitromin on both the nucleus and the cytoplasm of ascites-tumor cells are discussed.

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PLATE 26

Mitotic abnormalities in hepatoma 129(P) ascites-tumor cells, 24 to 48 hours after a single intraperitoneal injection of 50 γ per gm. of Nitromin. Acetic orcein. × 1200 (Pressed out in order to improve nuclear detail.)

FIGURES 1 and 2.—Chromosome-bridge formation, acentric fragmentation, and coagulation at anaphase.

FIGURE 3.—Coagulated chromosome bridge and cytoplasmic division which occurred independently.

FIGURE 4.—Chromosome-bridge formation in a tetrapolar anaphase.

FIGURES 5 and 6.—Examples of coagulation and scattering of chromosomes

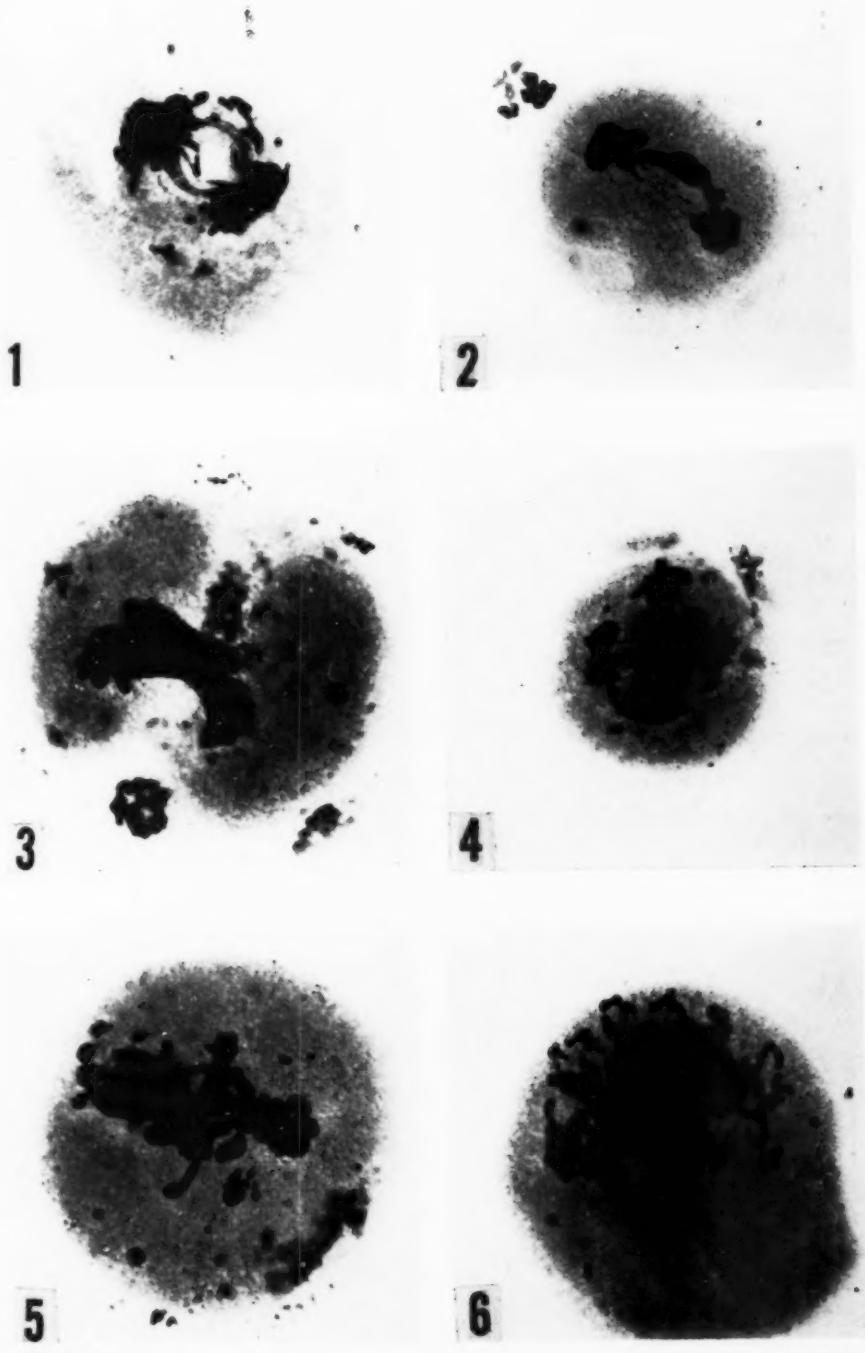

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PLATE 27

FIGURES 7 and 8.—Further examples of coagulation and scattering of chromosomes.

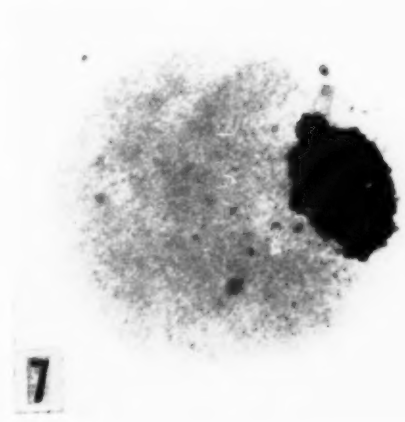
FIGURE 9.—Untreated hepatoma 129(P) ascites-tumor cells showing metaphase and early telophase figures. Giemsa. × 900

FIGURE 10.—Untreated hepatoma 129(P) ascites-tumor cells 5 days after inoculation. Giemsa. × 450

Mitotic abnormalities in hepatoma 129(P) ascites-tumor cells several days after intraperitoneal injection of Nitromin.

FIGURE 11.—Seventy-two hours after injection of 50 γ per gm. of Nitromin. *Note* appearance of mononucleate and binucleate giant cells and of cells in karyorrhexis. Acetic orcein. × 250

FIGURE 12.—Six days after injection of 50 γ per gm. of Nitromin. A single giant cell showing scattering and rounding up of chromosomes. Acetic orcein. × 400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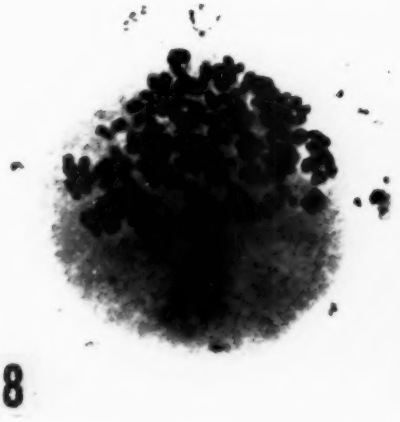
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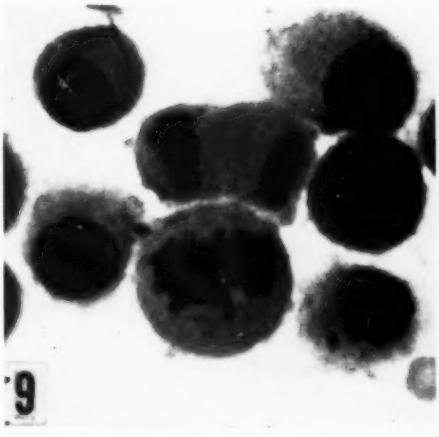
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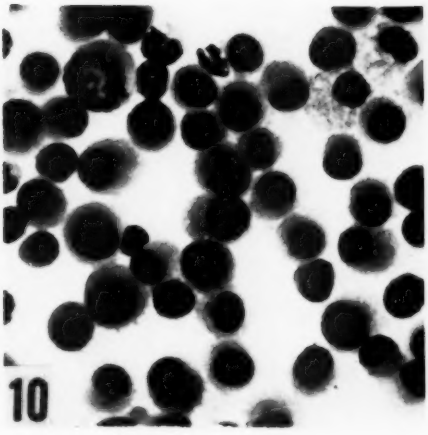
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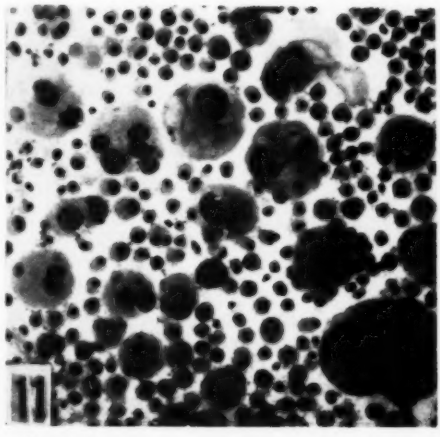
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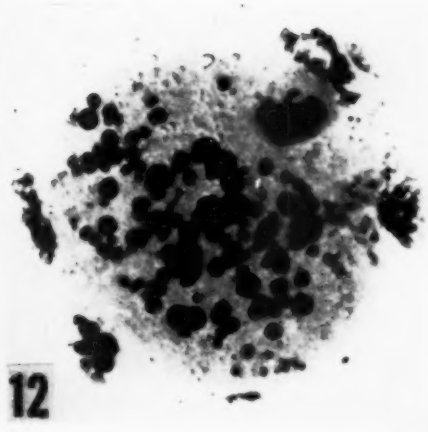
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Incidence of Neoplasms and Other Late Lesions in Mice Protected against Lethal X-Ray Doses by Spleen Homogenate [1,2,3]

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One of the most characteristic long-term effects of ionizing radiations on mammals is the increased occurrence of both malignant and benign neoplasms. The subject has been extensively documented and reviewed in the literature (*1–3*). The carcinogenic effect of ionizing radiation may be the result of a direct neoplastic transformation of the affected cells leading to malignant growth; or tumor induction may occur by some indirect mechanism. Radiation-induced tumors of the skin and bone are considered to be examples of direct action, while ovarian tumors, mammary tumors, and leukemias may be brought about by indirect means (*4*). Experimental data, particularly those of Kaplan (*3*) have provided strong evidence for the view that leukemogenesis in mice following exposure to ionizing radiation is mediated through an indirect mechanism, involving complex interactions among irradiated and nonirradiated tissues of the hematopoietic and endocrine systems.

The development of post-radiation protective agents, such as splenic homogenates and bone-marrow cell suspensions, has afforded a rather unique opportunity to study long-term effects of whole-body irradiation after exposure to otherwise lethal dose levels.

Previous reports from this laboratory (*5,6*) have provided experimental techniques and data on the protective effect of isologous mouse-spleen homogenates adminstered after whole-body X irradiation in the lethal and immediately supralethal dose ranges. The restorative, *i.e.*, post-protective, action of spleen homogenate has been characterized in terms of the following criteria: 30-day survival, body-weight recovery (*5*), spleen-weight involution and recovery (*6*), hematopoietic regeneration as measured by deoxyribonucleic acid (DNA) content and concentration of the spleen (*7*), and by the incorporation of C^{14}-labeled sodium formate into DNA purines of bone marrow and spleen (*8*). The purpose of this

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paper is to present the long-term findings in X-irradiated mice that survived as a result of treatment with spleen homogenates and were followed until natural death.

Material and Methods

The irradiation procedure and the pertinent data on the characteristics of the 250 KVP X radiation from the Westinghouse Therapy Unit employed have been documented in previous reports (*5,6*).

The mice used were LAF_1 hybrids, bred and raised in the animal colony of this laboratory. The data to be presented here constitute a collation of experiments on animals which had been exposed to lethal whole-body X irradiation in connection with spleen-homogenate post-protection experiments carried out over the 10-month period between December 1953 and October 1954. All the mice had received a single whole-body exposure of 250 KVP X rays at approximately 13 weeks of age. The dose, as measured in air with a Victoreen r-Chamber, ranged from 790 r to 820 r, not including the back-scatter contribution (approximately 15%).

Radiation exposure was followed within 1 to 3 hours by a single intraperitoneal injection of mouse-spleen homogenate, equivalent to 50 mg. fresh wet weight of spleen. The donor spleens were taken from 1-week-old LAF_1 mice, and the homogenates were prepared, as described previously, in an isotonic sucrose-salt solution containing adenosinetriphosphate (*9*) and 0.1 mg. per ml. dextrose. A total of 138 spleen-homogenate-treated survivors from these experiments were maintained and observed until natural death in order to study the long-term effects. Each experiment included irradiated control mice treated by injection with sucrose-salt alone or with heat-inactivated spleen homogenate (*10*). These control irradiated animals all died within 30 days after exposure.

A group of 658 normal LAF_1 mice of both sexes served as the nonirradiated control population and have been followed simultaneously with the irradiated animals. To date, 167 of these mice have been examined at death.

All the mice were maintained in standard galvanized metal cages, 8 to 10 per cage, and housed in an air-conditioned animal room. The diet was Purina laboratory chow, and tap water was allowed *ad libitum*. Routine autopsies were performed on all animals, and representative tissues were taken for microscopic examination.

Results

Malignant tumors.—The data on incidence of neoplasms in the lymphoma-leukemia group and of other malignant tumors in the nonirradiated and irradiated populations are summarized in table 1. It is apparent that no tumors appeared in either population before the age of 12 months. Among the nonirradiated mice dying at 12 to 24 months of age, there were 29 tumors of the reticular tissues ("leukemias"), while only 1 reticular tumor occurred in the X-irradiated, spleen-homogenate-treated group. On the other hand, nonlymphomatous malignant tumors were almost 3

times as common in the irradiated mice as in the nonirradiated animals of the 12-to-24-month age group. The mean age at death of the 126 X-irradiated mice in this group was 18.5 months and of the 130 nonirradiated controls, 21 months. No irradiated mice in this series have survived beyond 24 months, and thus far, the oldest nonirradiated control mice examined have been 28 months of age. In the small 25-to-28-month age group of controls, there has been a striking increase in both the leukemic and nonleukemic malignancies.

TABLE 1.—*Effect of spleen-homogenate injection on incidence of tumors in X-irradiated mice*

Treatment	Sex	Age at death (*months*)	No. of mice	Lymphoma-leukemia		Other malignant tumors	
				No.	Percent	No.	Percent
800 r + spleen homogenate		4–11	12	0	0	0	0
	Male	12–18	8	0	0	2	25
	Female	12–18	46	1	2	5	11
	Male	19–24	8	0	0	1	12
	Female	19–24	64	0	0	8	12
	TOTAL	12–24	126	1	1	16	13
Nonirradiated		4–11	2	0	0	0	0
	Male	12–18	5	1	20	0	0
	Female	12–18	6	1	17	0	0
	Male	19–24	74	10	14	1	1
	Female	19–24	45	17	38	5	11
	TOTAL	12–24	130	29	22	6	5
	Male	25–28	13	5	38	1	8
	Female	25–28	22	9	41	4	18

The great majority of hematopoietic malignancies in the nonirradiated animals were widely disseminated lymphomas involving abdominal lymph nodes, spleen, liver, and occasionally the bone marrow. Neoplastic cells in the peripheral circulating blood were not regularly observed. Reticulum-cell tumors outnumbered the lymphosarcomas, with the accompanying leukemic process, when present, being monocytic rather than lymphocytic. Two thymic lymphomas were noted in the nonirradiated mice, with the lesion limited to the thymus and adjacent structures. The single lymphoma occurring in the irradiated group was also of this thymic type. One case of granulocytic (myelogenous) leukemia was found in the nonirradiated group.

With respect to nonleukemic malignancies in the irradiated mice, mammary carcinoma was the most common epithelial tumor observed (3 cases); 1 Harderian-gland carcinoma was noted. No pituitary or thyroid tumors were observed. Of the connective-tissue tumors, osteogenic sarcoma (3 cases) was the most common. In the nonirradiated control

animals, only 2 carcinomas were observed, while subcutaneous and uterine sarcomas accounted for most of the miscellaneous malignancies.

Ovarian and pulmonary tumors.—The incidence of ovarian and pulmonary adenomas is presented in table 2. The induction of ovarian tumors in mice by irradiation has been observed and studied previously by various investigators (*11,12*), and the high incidence seen here is in agreement with their findings.

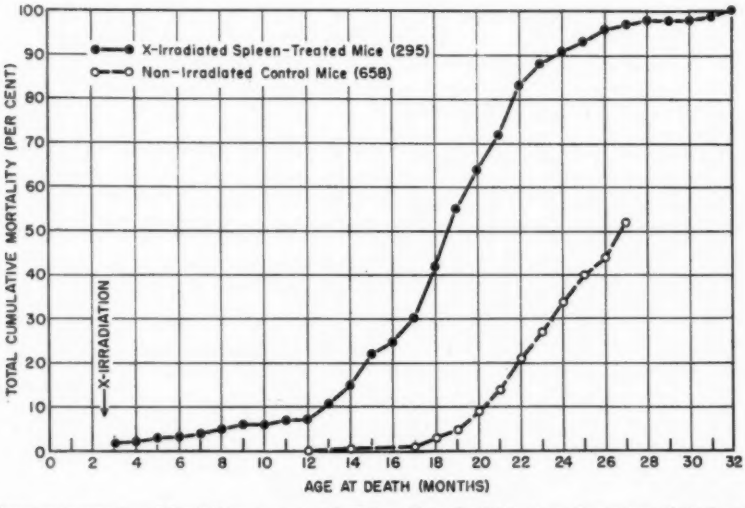
TABLE 2.—*Occurrence of ovarian and pulmonary adenomas in X-irradiated and nonirradiated mice*

Treatment	No. of mice*	Age at death (*months*)	Ovarian adenomas		Pulmonary adenomas	
			No.	Percent†	No.	Percent
800 r + spleen homogenate	12	4–11	0	0	0	0
	126	12–24	45	60	13	10
Nonirradiated	2	4–11	0	0	0	0
	130	12–24	4	8	26	20
	35	25–28	0	0	4	12

*Males plus females.
†Percent of females.

The relatively greater incidence of pulmonary tumors in the nonirradiated mice is of interest. In both groups, a small number of the adenomas had undergone malignant change, including several with distant metastases.

Longevity.—The data on the time-course of long-term mortality in X-irradiated, spleen-homogenate-treated mice and in nonirradiated control mice are plotted in text-figure 1.



TEXT-FIGURE 1.—Mortality curves of X-irradiated (800 r) and nonirradiated mice.

The control mortality curve is based on those of the 658 nonirradiated mice which have died to date. It is apparent that only one third of this group were dead by 24 months of age. Thus far, this curve is in close agreement with that of Furth's nonirradiated controls (*11*).

The mortality curve on the X-irradiated mice is based on a group of 295 LAF_1 mice exposed to 790 to 810 r and injected with spleen homogenate, as described above. The experiments from which these data were obtained were carried on separately from, and at an earlier time than, those affording the tumor-incidence data. The cumulative-mortality curve is a composite of the curve for males and females. In general, the female mice died 2 to 3 months *earlier* than the males, whose mean age at death was 20 months. Less than 10 percent of either sex survived beyond 24 months. A tabulation of the longevity data of the present report as compared to that of Upton and Furth (*13*) is given in table 3.

TABLE 3.—*Longevity of X-irradiated LAF_1 mice*

Treatment	No. of mice	Sex	Mean age at death (*months*)	Median age at death (*months*)
I.*				
800 r + spleen homogenate	100	M	20	21
	195	F	18	18
	295	M+F	19	19
Nonirradiated	658	M+F	27†	—
II.‡				
812–841 r (LD90)	220	M	19	
	220	F	14	
759–785 r (LD60)	220	M	19	
	220	F	16	
Nonirradiated	310	M	30	
	310	F	28	

*Data from our experiments.
†Age at which 50 percent of the mice had died.
‡Data from Upton and Furth (*13*).

Nephrosclerosis.—The extremely high incidence of sclerotic kidney lesions in the irradiated mice of the present series is seen from the data in table 4. These lesions, illustrated in figures 1 and 2, were characterized histologically by glomerular and arteriolar scarring, as well as dilation and atypical proliferation of the renal tubules. Inflammatory cells were inconspicuous. The nephrosclerosis seen here appears to be identical histologically with that previously described by Furth *et al.* (*11*) in LAF_1 mice exposed to ionizing radiation from a nuclear detonation without post-protection measures. The incidence in the present experiments is comparable to that observed in those of Furth's mice which received radiation doses above 500 r. In both series of experiments the incidence of nephro-

sclerosis in the control nonirradiated populations was less than 5 percent, even in the older age groups. Since Furth's data suggest a threshold dose of 500 r for the production of nephrosclerosis, this pathologic entity should be distinguished from the renal amyloidosis and papillonecrosis described by Heston (*14*) and by Hollcroft *et al.* (*15*) as occurring spontaneously in old nonirradiated mice of the LAF_1 and other strains. The latter disease generally occurs in mice beyond 24 months of age.

Table 4 presents also a breakdown of the nephrosclerosis-incidence data with respect to age at death. Comparison with the time-mortality curves in text-figure 1 demonstrates the striking rise in incidence of this disease during the period when the death rate, in terms of total numbers of mice dying, is increasing most rapidly. These data, as well as the pathologic findings at autopsy, imply that nephrosclerosis is a major cause of death in the irradiated mice. This conclusion is supported by preliminary laboratory evidence of impaired kidney function in these animals. In 3 irradiated mice 18 months of age, the urinary protein level was 3 to 4 mg. per ml. urine.

TABLE 4.—*Incidence of nephrosclerosis in X-irradiated mice (800 r) injected with spleen homogenate*

Treatment	Age at death (*months*)	No. of mice	Nephrosclerosis	
			No.	Percent
800 r + spleen homogenate	<12	12	1	8
	12–13	8	2	25
	14–15	9	2	22
	16–17	20	11	55
	18–19	42	38	90
	20–21	34	30	88
	22–24	10	10	100
Nonirradiated	4–11	2	0	0
	12–24	130	5	4
	25–28	35	1	3

Graying.—Depigmentation of the coat is a rather constant phenomenon seen in animals exposed to ionizing radiation (*16*). Furth *et al.* (*11*) observed graying in LAF_1 mice as early as 3 months after exposure to radiation from a nuclear detonation.

In the present group of X-irradiated mice treated with spleen homogenate, graying of the coat was first observed 2 months after exposure. The pattern of graying over the body was spotty and uneven at first but became diffuse by 9 months.

Discussion

The marked decrease in lymphoma incidence in the X-irradiated mice treated with spleen homogenate, as compared with nonirradiated control mice, is even more striking when compared with the data on late effects of Furth *et al.* (*11*) in a large series of mice of the same strain (LAF_1) exposed to ionizing radiation from a nuclear detonation. Furth's animals

were exposed to radiation doses ranging from 192 to 932 r and received no therapeutic treatment. In the animals surviving the highest dose levels (812 to 932 r — LD94 at 30 days, and 759 r — LD83) the incidence of ovarian adenomas and nephrosclerosis and the life span were in the same range as those for the irradiated, spleen-homogenate-treated mice reported here. However, Furth *et al.* observed an *increased* incidence of both thymic and generalized lymphomas, particularly thymic, as compared with non-irradiated controls of the same age. The lymphoma incidence in Furth's nonirradiated mice was quite similar to that observed in our nonirradiated controls. Therefore, it would appear that administration of spleen homogenate after otherwise lethal whole-body X irradiation in mice exerts a definite protective effect not only against acute (*i.e.*, 30-day) radiation death, but also against radiation-induced leukemia. Moreover, it is worthy of note that the injection of spleen homogenate did *not* influence the occurrence of nonlymphomatous malignancies, ovarian adenomas, or nephrosclerosis. Nor did it appear to reduce the degree of graying observed. The results, therefore, point up the *specific* aspect of post-radiation protection by spleen-homogenate administration. Restoration by the spleen "factor" apparently involves certain tissues of the hematopoietic system only, so that radiation-induced long-term effects in these tissues, such as leukemia, are specifically inhibited.

The parallelisms between Furth's longevity, nephrosclerosis, and ovarian-adenoma data and those reported here are of considerable interest, since they indicate that late effects of X irradiation are essentially identical with those from exposure to a nuclear detonation. The failure to observe pituitary adenomas in the present experiments is most probably due to the fact that all animals died within 20 months after irradiation. Furth *et al.* (*11*) have pointed out the extremely long latent period of these tumors.

Evaluation of the influence of spleen-homogenate injection on longevity is complicated by the fact that this treatment may result in shifts in the relative importance of the various causes of (late) death. Thus, irradiated mice, which without spleen treatment would have succumbed to leukemia, become candidates later for fatal sclerosis of the kidneys. Comparison of the present longevity data and those of Furth *et al.* suggests that the mean life span of irradiated female mice, surviving mid-lethal doses without post-treatment, was less (by approximately 2 months) than the lower 95 percent limits of error calculated for the life span of the irradiated female mice treated with spleen homogenate.

The possible mechanism for the apparently specific reduction of radiation-induced lymphoma by spleen-homogenate treatment is suggested by the experimental work of Kaplan and Brown and of Lorenz *et al.* The development of lymphoid tumors of the thymic type, which occurs in high incidence in whole-body X-irradiated mice of the C57BL strain, is greatly reduced by shielding the thigh (*17*) or the exteriorized spleen (*18*) during radiation exposure or by the injection of bone-marrow cell suspensions from nonirradiated C57BL mice (*19*). These measures have, in common

with spleen-homogenate injection, the property of promoting or stimulating earlier regeneration of hematopoietic and lymphoid tissues, including thymus, following their initial involution after radiation (*20,21,7*). Indeed, it is now generally recognized that the radiation-protection activity of mouse spleen, as well as bone-marrow cell suspensions, is referable to its content of hematopoietic elements, particularly those of the myeloid series (*22–24*). It is to be noted, furthermore, that the injection of *thymus* cell suspensions (*20*) or *thymus homogenates* (*10*) does not substitute for the required myeloid elements in protection against acute radiation death (*9,10*) or in promotion of thymus regeneration (*23*). It would appear probable, then, that induced accelerated regeneration of thymus and lymph nodes does not occur through primary repopulation by injected thymus cells. And, since unpublished histologic studies in this laboratory indicate that regeneration of the mouse thymus is not due to direct repopulation by injected bone-marrow cells *as such* (*25*), it seems plausible that induced thymic regeneration and the inhibition of thymic-lymphoma production are the resultant of an indirect mechanism secondary to regeneration of the bone marrow and spleen.

The decreased incidence of lymphomas in the irradiated, spleen-treated mice as compared with the nonirradiated controls is apparently not due to an age or sex difference between these groups, since the mean age of the control mice in the 12-to-24-month age group was only 2½ months greater than that of the irradiated mice in the same age bracket. Furthermore, the lymphomas in the control mice were almost twice as prevalent in the females as in the males, and the irradiated, spleen-treated mice were mostly females.

Summary

A total of 138 LAF_1 mice, protected against lethal doses of X ray (800 r) by post-irradiation injection of spleen homogenate, were followed until natural death.

Leukemias and lymphomas occurred in less than 1 percent of the irradiated mice and in 22 percent of a comparable group of nonirradiated controls of the same age. The incidence of other malignant tumors, ovarian adenomas, and nephrosclerosis was markedly increased over the nonirradiated controls.

The mean age at death of the irradiated, spleen-homogenate-treated mice was 19 months, 8 months earlier than comparable nonirradiated controls. Nephrosclerosis was a major cause of late death in the irradiated mice.

The apparent specific action of spleen-homogenate post-protection in preventing radiation-induced leukemia appears to be associated with its promotion of more rapid regeneration of the damaged hematopoietic system, including the lymphoid tissues.

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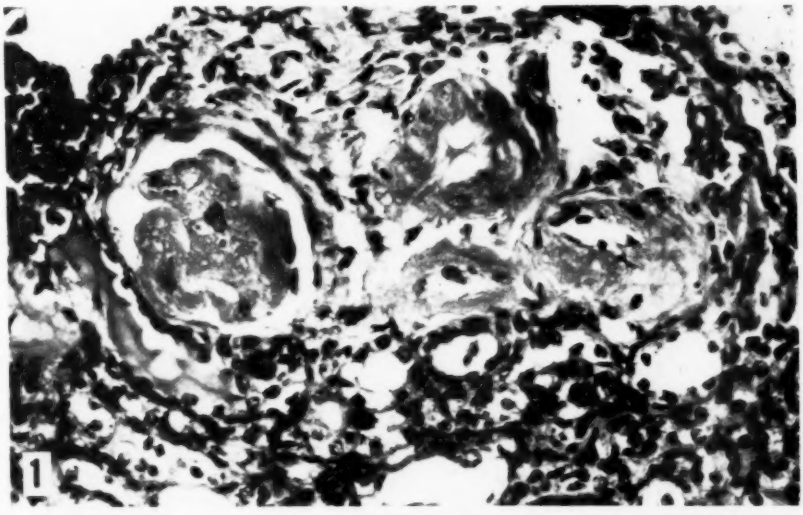
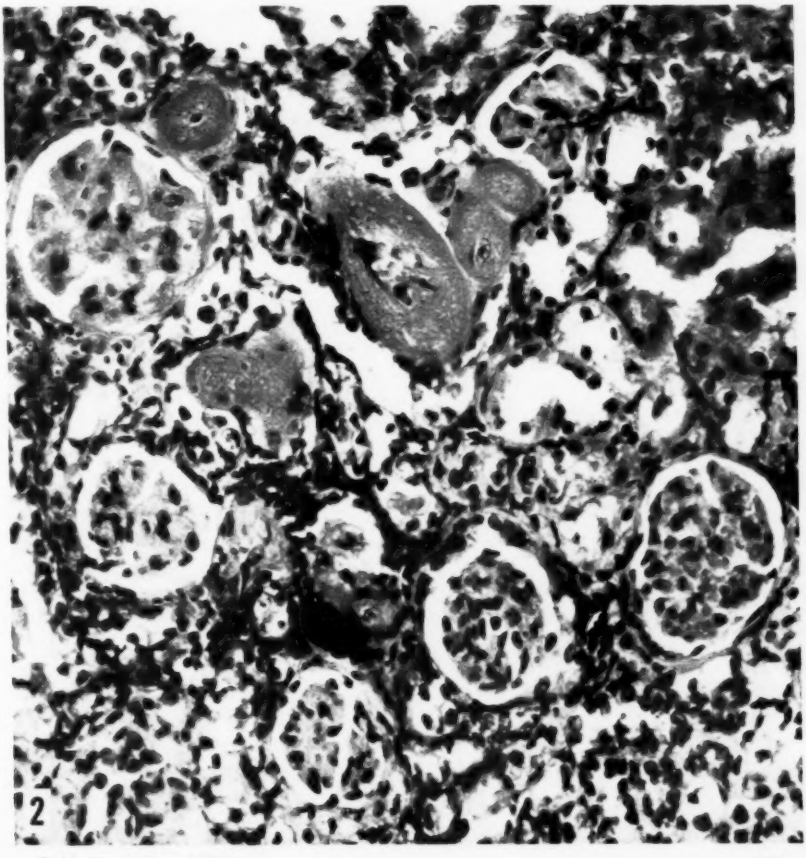
PLATE 28

FIGURES 1 and 2.—Typical arteriolar and glomerular sclerosis in LAF_1 mouse 13 months after X irradiation (800 r) and injection of spleen homogenate. Hematoxylin and eosin. × 375

Photomicrographs by Mr. Victor Duran.





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Observations on a Leukemic Cell Variant in Mice [1]

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The observations presented in this paper were made during the course of an investigation of the effect of amethopterin administration on the host-tumor relationship, using a variant of mouse leukemia L1210 AmD.[3] This subline was reported (*1*) to be not only resistant to amethopterin but also dependent upon it for optimal growth. The suggestion was made that these leukemic cells had acquired the ability to utilize amethopterin (*1*). The data reported here confirm the earlier observation (*1*) that the AmD leukemia employed shows little or no sensitivity to amethopterin. Evidence is presented that when an infectious agent is associated with leukemia the interrelationships of infectious agent, leukemic cells, host, and drug may duplicate the appearance of dependence of the leukemic cells on amethopterin.

Methods

The L1210 AmD leukemia and the L1210 leukemia sensitive to amethopterin were carried in DBA/2J and CDBA [4] hybrid male mice. The L1210 AmD stock mice were treated with amethopterin [5] (3 mg. per kg.) every other day. The experiments were conducted mainly in CDBA male mice 3 to 4 months old. Other strains of mice were employed where indicated. Transplantation of the tumor and infectious agent (*see* section on Results)

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[1] Received for publication May 11, 1956.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] The authors wish to thank Dr. Lloyd Law for supplying us with this tumor on several occasions. A strain DBA mouse bearing the AmD tumor (no generation number) was first received on February 4, 1953. It was transplanted the next day from the original stock into 2 groups of mice, those treated with amethopterin and those receiving no amethopterin. Mice which received no treatment showed signs of illness. The inoculum for the experiment in table 1 was obtained from the mice which received amethopterin and showed no signs of illness. This tumor was discarded when the laboratory was moved from Baltimore to Bethesda.

A CDBA mouse bearing L1210 AmD, Line F_X—41st generation, was received October 11, 1955. We were informed that this mouse had been given 3 mg. per kg. of amethopterin, 50,000 units of penicillin, and streptomycin every other day. The tumor was transplanted on the day of receipt, and all mice received amethopterin. In the following generation mice which did not receive amethopterin showed evidence of illness. This tumor subline was employed for the experiments in tables 2, 3, and 4.

A tumor was also received on May 3, 1955. Bacteriologic examination revealed the presence of a gram-negative, spore-forming rod; this tumor stock was discarded immediately.

[4] (BALB/cAn × DBA/2J)F_1.

[5] Amethopterin was kindly provided by Dr. J. M. Ruegseggar, of the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N. Y.

was ordinarily accomplished by injecting a 0.1 ml. saline suspension of spleen into the right thigh. Amethopterin was prepared in aqueous 2 percent sodium bicarbonate and injected subcutaneously in the axillary region in the constant volume of 0.01 ml. per gm. of body weight.

Aseptic procedures were employed throughout, and, in experiments designed to separate the tumor from the infectious agent, additional precautions were taken to avoid cross-contamination. The animals were weighed daily and their general condition was recorded. The local tumors at the site of leukemic inoculation were palpated and the animals were observed for survival time. Tumor diameters, when given, were obtained either as an average of the 3 diameters of the tumor itself or as the diameter of a bead closest to the tumor in size.

Results

In preliminary experiments employing AmD leukemia [6] it was observed that the tumor at the site of leukemic inoculation responded to amethopterin as previously reported (*1*). Two typical experiments are summarized in tables 1 and 2.

In the first experiment (table 1), it was observed that the tumor (L1210 AmD) at the site of leukemic inoculation grew more rapidly and the survival time of the host was somewhat shorter in mice treated with amethopterin than in untreated controls. In addition to the slower growth of tumor and longer survival time of untreated mice the mice showed evidence of illness and displayed only moderate weight gains, although many lost weight. The mice eventually recovered from this illness, the tumor growth rate then appeared to increase, and finally all mice died with tumors of moderate size. Mice treated with amethopterin showed little or no evidence of illness and usually gained weight.

In the second experiment (table 2), untreated mice given L1210 AmD showed evidence of very severe illness and all lost weight. Most of these mice died early; none showed definite signs of tumor at the time of death. Amethopterin-treated mice did not show evidence of illness and there was little or no weight loss. In all cases the tumor grew progressively, eventually causing death. Here, in contrast with the first experiment, the increase in size of the local tumor was accompanied by an increase in survival time. Mice inoculated with the amethopterin-sensitive strain of L1210, but not given amethopterin (table 2), gained weight and the tumor grew progressively. The mice appeared to be in excellent condition until shortly before death. Treatment with amethopterin caused marked retardation of tumor growth and a corresponding increase in survival time.

In other experiments with L1210 AmD leukemia the response of untreated mice varied from the moderate illness of the first experiment to the severe illness noted in the second experiment. These observations suggested the presence of an infectious agent associated with and capable of retarding

[6] See footnote 3.

TABLE 1.—*Effect of amethopterin on AmD subline of leukemia L1210 in DBA/2J mice*†*

Age of mice (*weeks*)	No. of mice	Amethopterin treatment‡ (*mg./kg.*)	Average animal weight change (*gm.*)		Average tumor diameter § (*mm.*)		Average survival time (*days*)
			Day 7	Day 10	Day 7	Day 10	
32	8	0	+0.4	+1.1 (6)	11.7	12.6 (6)	12.2
	9	3	+1.7	+2.4 (8)	13.0	15.2 (8)	11.6
21	10	0	−0.7	−2.7	12.1	13.3	11.9
	10	3	+2.3	+2.4 (9)	12.2	14.1 (9)	10.9
8	10	0	+0.3	+0.9	10.7	13.3	11.6
	10	3	+2.2	+2.3 (9)	12.2	15.0 (9)	11.0

*Figures in parentheses indicate the number of mice on which each average is based. For data on weight change and tumor diameter these represent surviving animals.

†Inoculum: L1210 AmD, 2.1 × 10^5 cells per mouse.

‡Treatment schedule: Days 2, 4, 6, 8.

§The relative increase in the size of L1210 AmD tumors produced by amethopterin treatment would appear much greater if average tumor weights rather than average tumor diameters were obtained.

TABLE 2.—*Comparison of effect of amethopterin on L1210 AmD and amethopterin-sensitive L1210 leukemias in CDBA mice**

Inoculum	No. of mice	Amethopterin treatment† (*mg./kg.*)	Average animal weight change (*gm.*)		Average tumor diameter (*mm.*)		Average survival time (*days*)
			Day 7	Day 10	Day 7	Day 10	
L1210 AmD‡	4	0	−1.8	−5.0 (1)	§	§	10.0
	4	3	+0.5	0.0	7.5	12.8	12.2
Amethopterin-sensitive L1210‡	4	0	+0.8	+2.0	§	9.8	13.0
	4	3	+1.8	+2.0	§	§	37.0

*Figures in parentheses indicate the number of mice on which each average is based. For data on weight change and tumor diameter these represent surviving animals.

†Treatment schedule: Every 2 days beginning on day 0.

‡Inoculum: 0.1 cc. from mash containing 1 spleen per 10 cc.

§No definite tumors present at this time.

the growth of the leukemia.[7] They also suggested that amethopterin afforded protection against this agent. In the second experiment, the infection apparently was lethal to the mice. Treatment with amethopterin, by protecting against the lethality of the infection, increased survival time despite the resulting increase in tumor growth.

The possibility that an infectious agent is associated with the AmD leukemia was also indicated by the following observations. When the AmD leukemia was transplanted into another strain (A/LN) the mice became ill. In the first generation, some tumor growth, followed by regression, occurred at the site of leukemic inoculation. However, after serial subcutaneous transfer of the spleens of these mice to mice of the

[7] Preliminary studies in this laboratory suggested that the infectious agent was probably of a viral nature. Subsequently, a virus was isolated from this leukemia and identified by S. E. Stewart and V. H. Haas (personal communication). Viral agents associated with leukemia have been reported previously (2–4).

same strain, for 40 generations, the mice became ill consistently and always died in 7 to 10 days without evidence of tumor. Retransplantation of A/LN spleen to DBA/2J mice gave no evidence of tumor but the animals lost weight and died, apparently because of infection. Amethopterin treatment diminished the symptoms of infection, and the mice survived. It was evident from these observations that the infectious agent could be separated from the tumor and that its effects could be controlled by treatment with amethopterin.

Development of "Infection-Free" Leukemia

An attempt was made to free the tumor from infection by a method which previously had proved useful in this laboratory. Strain CDBA mice were inoculated intraperitoneally with the AmD leukemia. All became acutely ill by the 6th or 7th day and 1 died from infection. On the 13th day, the remaining mice appeared to have recovered from the infection and showed signs of developing intraperitoneal fluid. The fluid and spleens of these mice were used to inoculate the mice for the experiment summarized in table 3.

The results of this experiment (table 3, groups 1 to 7) indicate that the extent of infection had been reduced by intraperitoneal passage and that the tumor, under these circumstances, is relatively resistant to but not dependent upon amethopterin for optimal growth. The leukemic mice not treated with amethopterin remained in good health and gained weight, but their tumors grew progressively to a large size. There was no evidence of stimulation of tumor growth after treatment with amethopterin. Instead, tumor growth was somewhat retarded, and this was associated with loss of weight. The lower doses of amethopterin produced a slight increase in survival time; at the highest dose the mice lost considerable weight, and their survival time was diminished.

However, there was evidence in the above experiment that some infection was still present. At levels of tumor inoculum at which the mice did not develop leukemia (table 3, groups 10 to 12), they showed illness and weight loss. Also, DBA and A/LN mice (table 3, groups 14 and 15) receiving the initial concentration of inoculum became ill. In each of these groups half the mice died either tumor-free or with only small tumors. However, C57BL/6JN mice (table 3, group 13) showed resistance to both infection and tumor, and no deaths occurred.

In other experiments, the infection was suppressed by passage of the leukemia through mice which had recovered from and were evidently immune [8] to the infection. These were CDBA mice which had received a sufficiently low inoculum of the AmD leukemia that, although the leukemia did not take, the mice showed transient illness. The AmD tumor, transplanted into nonimmune mice after prior passage through immune animals, behaved like a tumor resistant to amethopterin, showing no evi-

[8] The spleens of recovered mice apparently did not transmit the infection. Also, such mice did not become ill when challenged with the infectious agent. A similar immunity was found in infected mice which had been treated with amethopterin.

TABLE 3.—*Loss of dependence on amethopterin of AmD subline of leukemia L1210 following I.P. passage**

	Group	Mouse strain	No. of mice	Tumor inoculum†	Amethopterin treatment‡ (*mg./kg.*)	Average animal weight change (*gm.*)			Average tumor diameter (*mm.*)			Average survival time (*days*)
						Day 9	Day 11	Day 14	Day 9	Day 11	Day 14	
Experimental	1	CDBA	10	1	3.12	−3.2(8)	−4.7(6)	−6(1)	8.2(8)	9.8(6)	10(1)	11.9
	2		10	1	1.86	−0.4	−1.4(9)	−3.0(2)	10.7	11.9(9)	11.5(2)	13.2
	3		10	1	1.11	−1.5	−1.3	−2.1(7)	9.2	11.1	12.4(7)	14.6
	4		10	1	0.67	−2.5	−1.2	+1.0(8)	7.7	10.5	12.4(8)	15.3
	5		10	1	0.40	−1.1	+0.3	+1.0(6)	7.9	10.9	13.0(6)	14.9
Untreated controls	6	CDBA	10	1	0	+0.1	+3.9	+5.5(2)	9.8	13.2	14.0(2)	14.2
	7		10	1	0	−0.5	+2.3	+3.7(3)	9.7	12.7	14.0(3)	14.4
Varying tumor inoculum	8	CDBA	6	0.1	0	−1.2	+0.2	+2.8	6.5	7.7	12.3	17.5
	9		6	0.01	0	−0.5	0.0	+0.2	§	5.7	9.0	18.8
	10		6	0.001	0	−0.8	−0.8	−1.7	§	§	§	20(1)
	11		6	0.0001	0	−0.5	−1.3	−1.8	§	§	§	22(1)
	12		6	0.00001	0	−0.3	−0.3	−0.5	§	§	§	‖
Others	13	C57BL/6JN	6	1	0	−0.7	+0.3	0.0	§	§	§	‖
	14	A/LN	6	1	0	−2.7	−2.5	−3.0(5)	¶	¶	¶	23.0(3)
	15	DBA/2J	6	1	0	−3.5(4)	−3.3(3)	−3(1)	8.5(4)	11.0(3)	13(1)	11.8

*Figures in parentheses indicate the number of mice on which each average is based if different from the initial number in the group. For data on weight change and tumor diameter these represent surviving animals; for data on survival time they represent mice dying prior to termination of the experiment (day 46).

†Inoculum: L1210 AmD from I.P. passage. 1=2.8 × 10^6 cells.

‡Treatment schedule: Daily from day 3.

§No definite tumors present at this time. In group 9 all mice eventually developed tumors; in group 11 a tumor eventually appeared, resulting in death of a mouse on day 22.

‖No deaths occurred during the period of the experiment.

¶Four tumors occurring in this group eventually regressed. Three mice in this group died, 2 of them with regressed tumors, 1 with no definite appearance of tumor at any time. With the amethopterin-sensitive L1210 leukemia in A/LN mice the typical pattern is one of appearance of tumors followed by regression, but deaths occur only occasionally.

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dence of dependence. Figure 1 illustrates the growth into a large tumor of the AmD leukemia in an immune mouse not treated with amethopterin.

One line of this "infection-free" tumor has been carried in nonimmune mice for 9 generations and has shown no evidence of dependence on amethopterin for optimal growth.

Development of "Tumor-Free" Infectious Agent

Initially, as indicated previously, the infectious agent had been dissociated from the leukemia by passage in A/LN mice. The agent was separated a second time by serial dilution of the leukemic inoculum—in CDBA mice to avoid any possibility of alteration of the infectious agent by passage through another strain of mice. The CDBA mice were injected subcutaneously with various dilutions of L1210 AmD spleen cells. Mice that received sufficiently dilute concentrations of the inoculum showed transient illness and did not develop leukemia during the period of observation (90 days). Several of these were sacrificed when symptoms of infection were evident and their spleen cells were inoculated into normal CDBA animals. The resulting "tumor-free" infectious agent has been carried by subcutaneous serial transfer of spleen for 9 generations. The mice have become ill consistently and many have succumbed to the infection. There has been no evidence of leukemia either among the survivors or among those dying from infection.

Recombination of Leukemia and Infection

The "infection-free" tumor and the "tumor-free" infectious agent were inoculated into mice individually and in combination, and comparisons were made with and without amethopterin therapy (table 4).

The tumor alone showed no dependence upon amethopterin for optimal growth; however, it did show relatively great resistance to amethopterin. Nevertheless, treatment with the drug resulted in a slight increase in survival time and minimal reductions of weight gain and tumor growth (table 4, groups 1 and 9).

Inoculation of the infectious agent alone (table 4, groups 14 to 16) did not result in tumor growth. As the concentration of the infection inoculum was increased, the symptoms of infection, including weight loss, increased progressively, as well as the proportion of mice dying from infection. Treatment with amethopterin (table 4, groups 6 to 8 and 14 to 16) reduced the symptoms of infection, including weight loss. Mortality from infection was completely prevented by treatment with amethopterin.

In groups that received both tumor and infectious agent (table 4, groups 10 to 12), the length of time for appearance of the tumor was retarded and the ultimate size of the tumors was reduced. These effects were more pronounced at the higher concentrations of infectious agent. Despite some early deaths in these groups there was an increase in average survival time over uninfected leukemic controls (table 4, group 9). Amethopterin treatment (table 4, groups 2 to 4 and 10 to 12) decreased the severity of the infection and the accompanying loss of weight and

TABLE 4.—*Duplication of leukemic dependence on amethopterin in CDBA mice through recombination of tumor and infection**

Group	No. of mice	Tumor inoculum†	Infection inoculum‡	Amethopterin§ (*mg./kg.*)	Average animal weight change (*gm.*)					Average tumor diameter (*mm.*)				Average survival time (*days*)
					Day 7	Day 9	Day 11	Day 13	Day 19	Day 7	Day 9	Day 11	Day 13	
Tumor; amethopterin; varying infection inoculum														
1	10	1	0	3	+0.7	+0.4	+2.0	+0.8(4)	‖	6.8	10.8	13.8	14.2(4)	13.4
2	10	1	0.0001	3	+0.9	+0.7	+2.6	+3.2(6)	‖	5.9	10.5	13.6	14.5(6)	14.2
3	10	1	0.01	3	+0.6	−0.7	−2.3	−3.9(9)	‖	6.1	10.8	12.0	12.7(9)	14.7
4	10	1	1	3	−0.3	−1.4	−3.2	−4.4(8)	‖	6.4	10.7	11.7	13.0(8)	14.5
No tumor; amethopterin; varying infection inoculum														
5	10	0	0	3	+0.8	−0.2	+0.6	−0.1	+0.8	¶	¶	¶	¶	**
6	10	0	0.0001	3	+0.3	−0.8	−0.8	−1.1	+0.2	¶	¶	¶	¶	**
7	10	0	0.01	3	+0.5	−1.0	−2.3	−3.8	−0.8	¶	¶	¶	¶	**
8	10	0	1	3	−0.4	−1.6	−3.8	−4.2	−1.3	¶	¶	¶	¶	**
Tumor; no amethopterin; varying infection inoculum														
9	10	1	0	0	+1.9	+1.4	+3.4	+2.5(2)	‖	6.7	12.0	14.5	15.0(2)	12.9
10	10	1	0.0001	0	+0.7	+0.2	+1.8	−0.7(6)	+5(1)	6.5	11.9	13.3	13.3(6)	14.7
11	10	1	0.01	0	−1.3	−3.8	−5.2(9)	−4.4(9)	‖	¶	5.8	6.8(9)	11.2(9)	14.8
12	10	1	1	0	−2.8	−5.0(9)	−6.8(9)	−6.0(8)	‖	¶	¶	¶	9.1(8)	14.6
No tumor; no amethopterin; varying infection inoculum														
13	10	0	0	0	+0.7	+0.5	+0.4	+0.8	+1.8	¶	¶	¶	¶	**
14	10	0	0.0001	0	+0.3	+0.1	+0.2	−0.1	+1.6	¶	¶	¶	¶	**
15	10	0	0.01	0	−1.9	−4.5	−5.4(9)	−4.2(9)	−4.3(9)	¶	¶	¶	¶	11(1)
16	10	0	1	0	−2.7	−5.5(8)	−6.3(7)	−5.6(5)	−6.2(5)	¶	¶	¶	¶	10.8(5)
Varying tumor inoculum; no amethopterin														
17	6	0.01	0	0	+0.2	+0.5	+0.5	+0.7	‖	¶	¶	6.0	9.3	17.8
18	6	0.0001	0	0	+2.2	+1.3	+1.7	+0.7	+2.8	¶	¶	¶	¶	22.5(2)

*Figures in parentheses indicate the number of mice on which each average is based if different from the initial number in the group. For data on weight change and tumor diameter these represent surviving animals; for data on survival time they represent mice dying prior to termination of the experiment (day 35).

†Tumor inoculum from "infection-free" L1210 AmD spleen mash. 1=0.1 cc. from mash containing 1 spleen per 10 cc.

‡Infection inoculum from "tumor-free" spleen mash. 1=0.1 cc. from mash containing 1 spleen per 10 cc.

§Amethopterin administered every other day from day 1.

‖No survivors on this day.

¶No definite tumors present at this time. In groups 11, 12, and 17 all mice eventually developed tumors, except those dying early from infection. In group 18, 2 mice eventually developed and died from tumor.

**No deaths occurred during the period of the experiment.

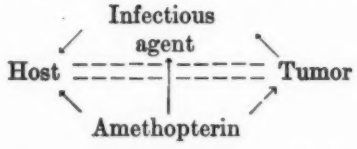
permitted increased growth of the tumor. Although treatment with amethopterin did not increase the survival time over that of comparable infected mice receiving no amethopterin, it did increase the survival time as compared with that of noninfected untreated leukemic mice (table 4, group 9).

Discussion

Previous studies have shown that the antileukemic specificity of action of a drug may be influenced by a number of factors pertaining to the host, the tumor, and the drug, such as: the time at which treatment is initiated; the number of treatments; the length of the interval between treatments; the total dose; the administration of a metabolite with an antimetabolite and the effect of time of administration of both, either alone or together; the number of leukemic cells; and the age and weight of the host. Any or all of these factors may influence the chemotherapeutic effectiveness of a drug (*5–11*). The current experiments indicate that the presence of an infectious agent is another factor which may influence the host-tumor relationship in chemotherapy.

Evidence was obtained that an infectious agent is associated with the amethopterin-dependent (AmD) variant of leukemia L1210 employed in these experiments. This variant had been reported to be resistant to, and dependent upon, amethopterin for optimal growth (*1*). It was possible to suppress the infection associated with the leukemia by intraperitoneal passage of the leukemia in nonimmune mice or by passage through immune animals. It was also possible to isolate the infectious agent from the leukemia, either by supression of the leukemia by passage through another strain of mice (A/LN) or by the administration of a subthreshold leukemic inoculum in CDBA mice. The "infection-free" tumor and "tumor-free" infectious agent were carried independently by serial passage of spleen in successive generations.

The following schema, based on observations of "infection-free" tumor, "tumor-free" infectious agent, and recombination of the 2, depicts the interrelationships of host, tumor, infectious agent, and drug:



(*a*) The infectious agent retards the growth of the leukemia. The extent to which the effect is direct or is mediated through the host has not been determined.

(*b*) The infectious agent is deleterious to the host, inducing illness, weight loss, and death.

(*c*) Amethopterin is relatively ineffective in retarding the growth of the AmD leukemia when the latter is free of infectious agent.

(*d*) Amethopterin toxicity to the host is a function of dosage.

(*e*) Amethopterin suppresses the deleterious action of the infectious agent on the host. It reduces the extent of illness and loss of weight and prevents death of the host.

It overcomes the antileukemic action of the infectious agent. As a result, amethopterin treatment, when the infection is present, leads to increased growth of the leukemia.

(*f*) The survival time of the host is subject to the interaction of infectious agent and drug in influencing the tumor and host.

Initial experiments with the original AmD leukemia reproduced results reported earlier (*1*). Treatment with amethopterin increased the rate of growth of the tumor at the site of inoculation. On recombination of the "infection-free" leukemia and the "tumor-free" infectious agent this observation was corroborated.

The current observations support the finding (*1*) that AmD-leukemia cells are relatively insensitive to amethopterin. They also demonstrate that biological phenomena which would result from true leukemic-cell dependence on a drug can be duplicated by indirect effects of an infectious agent. Thus, the observation that certain tumor cells grow better in animals receiving a chemical agent than in untreated animals may be explainable not necessarily by dependence of tumor cells on the compound, but by a more complex mechanism involving the suppression of the infectious agent by the compound, resulting in improved growth of the tumor. These data do not imply that the phenomenon of leukemic-cell dependence on a drug does not exist. They do suggest, however, that in investigations of dependence, the mechanism described here should be taken into consideration.

The current experiments may also have certain implications for tumor therapy. In previous studies (*6,8*) it was demonstrated that the antileukemic specificity of action of an antimetabolite may be enhanced by a metabolite by taking avantage of a differential protection for host and tumor. The results of this investigation suggest the possibility of employing antimetabolites alone or in conjunction with metabolites to control both naturally occurring infections and infections specifically administered for their anti-tumor effect.

Summary

An infectious agent was found to be associated with an amethopterin-dependent (AmD) variant of leukemia L1210 in mice. The leukemia and infectious agent were dissociated, carried independently by serial transfer of spleen, and recombined. The "infection-free" leukemia remained relatively resistant to amethopterin but showed no evidence of dependence on the antagonist for optimal growth. The "tumor-free" infectious agent retarded leukemic growth and was harmful to the host. Protection from the infection was afforded both host and tumor by amethopterin treatment; furthermore, since the tumor was resistant to amethopterin, an increase in tumor growth resulted. This increase in growth of the leukemia, resulting from the interaction of host, tumor, infectious agent, and drug,

duplicated the appearance of dependence of the leukemic cells on a-methopterin.

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PLATE 29

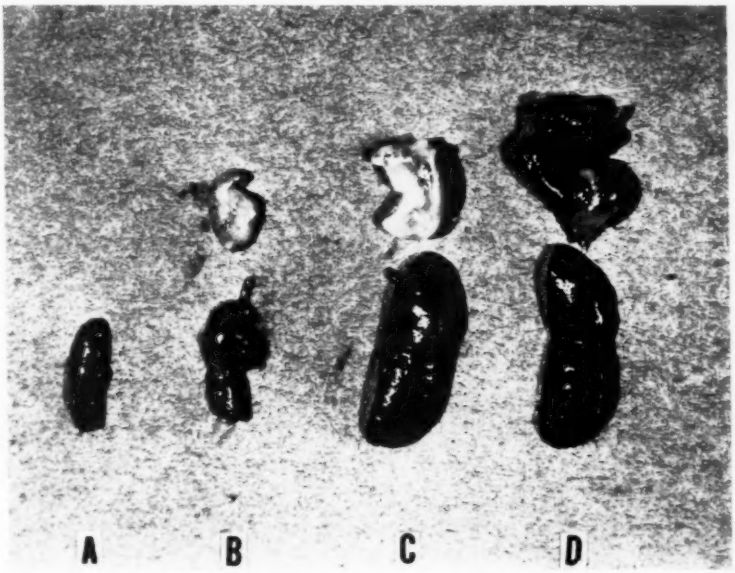
FIGURE 1.—All mice were CDBA F_1 males 17 weeks old. Leukemic mice all received the same inoculum of L1210 AmD 10 days before sacrifice.

(*A*) Spleen of normal mouse.

(*B*) Spleen and tumor of a mouse that received no amethopterin treatment. *Spleen:* small, irregularly shaped, with mottled appearance of infection. *Tumor:* small and pale.

(*C*) Spleen and tumor from mouse treated with 3 mg. per kg. amethopterin every other day. *Spleen:* large, swollen appearance with rounded margin. *Tumor:* moderately large and pale.

(*D*) Spleen and tumor from a mouse "immune to infection." *Spleen:* large, irregularly shaped, with rough margin. *Tumor:* large and congested in appearance.



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Recorded and Expected Mortality among the Japanese of the United States and Hawaii, with Special Reference to Cancer [1]

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Previous studies have presented evidence consistent with the view that Japanese experience a different risk for cancer of several sites from that observed among white populations. Thus, a relative excess of cancer of the stomach and a relative deficit of cancer of the breast have been noted in several studies of clinical and autopsy records (*1–5*). Mortality statistics, as reported by Segi, have confirmed this (*6,7*). In addition, one study of cancer mortality among the several racial groups in Hawaii has demonstrated similar differences (*8*).

The purpose of this study is to extend previous observations by comparing mortality recorded among Japanese residing in Hawaii and in the Continental United States with deaths expected among them had they been subject to the same rates prevailing among the white and nonwhite United States population. Such differences as may be observed with this procedure should less likely be due to different medical and death-certification practices than when similar comparisons are made between Japanese dying in Japan and Americans dying in the United States.

Method of Study

The figures for deaths among the Japanese of the United States and Hawaii which were attributed to malignant neoplasms were obtained from special tabulations provided by the National Office of Vital Statistics for the years 1949–52. These were compared with deaths expected among them on the basis of the 1950–51 (*9*) average annual death rates of the white and nonwhite (95% Negro) population of the United States. Expected deaths were obtained by multiplying the age-, sex-, and cause-specific United States rates by the corresponding population groups of the Japanese in Hawaii and the United States, as estimated for January 1, 1951. The results of these multiplications, summed for all age groups and multiplied by 4, thus represent the deaths from each cause expected to occur among the Japanese in the 4 years 1949–52. The number of observed deaths, divided by those expected, yields a ratio. This ratio,

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

multiplied by 100, gives a standardized mortality ratio (SMR), which expresses the Japanese mortality, age adjusted by the indirect method, as a percentage of the United States average annual death rate.

The confidence which may be placed in differences demonstrated by these procedures is to some extent influenced by the forces of mortality which cancer replaces, or those causes of death which may replace cancer. For this reason SMR's have also been computed for all causes of death and for deaths grouped in conventional broad causes. The latter SMR's, however, pertain only to the Japanese in the United States, since recorded deaths other than for cancer were not readily available for the Japanese in Hawaii.

Although it has been pointed out previously (*10*), it is not generally appreciated that the indirect method of age adjustment, which is employed in this study, does not always give the same results as obtained by the direct method. In some unusual situations, the 2 methods can, in fact, yield opposite results. For this reason the data of this study have been analyzed by both methods for those causes of death which were recorded in sufficient numbers to make valid age adjustments by the direct method. Since in each such comparison the 2 methods yielded similar differences between the races studied, only the SMR's derived from the indirect method of adjustment are presented here.

General Results

Table 1 presents the number of deaths recorded in 4 years among the Japanese residing in the United States by broad causes of death, together with the deaths expected on the basis of United States white and nonwhite rates and the corresponding SMR's. It is seen that the over-all age-adjusted mortality is less than either the white or nonwhite mortality and that the majority of the categories contribute to this deficit. The only causes for which Japanese experienced excessive mortality were (I) infective and parasitic diseases, compared to white rates for both sexes (due entirely to tuberculosis), and (II) neoplasms among Japanese males, compared to nonwhite male rates. There were a few causes for which mortality among United States Japanese did not differ significantly from that of United States whites, but only 2—(IV) diseases of blood-forming organs and (XIV) congenital malformations—in which there was no significant difference from the nonwhite as well as white rates. It is of interest that very few Japanese deaths are assigned to ill-defined causes or senility.

Mortality Attributed to Cancer

At the mid-point of time for which mortality data were available (Jan. 1, 1951), it is estimated that there were 186,601 Japanese residing in Hawaii and 142,446 residing in the Continental United States. Neither group alone is sufficiently large to produce very stable rates or ratios when cancer is divided by individual sites. It was therefore desirable to judge whether

TABLE 1.—*Deaths recorded among Japanese residing in the United States during 1949–52, by broad causes; deaths expected on the basis of age-specific rates observed in 1950 and 1951 among United States whites and nonwhites; and resultant standardized mortality ratios (SMR's)*

Broad cause of death	International list number	Males					Females				
		Number of recorded deaths	On basis of nonwhite rates		On basis of white rates		Number of recorded deaths	On basis of nonwhite rates		On basis of white rates	
			Number of expected deaths	SMR	Number of expected deaths	SMR		Number of expected deaths	SMR	Number of expected deaths	SMR
I Infective and parasitic	(001–138)	237	367	65*	112	212*	57	182	31*	40	142*
II *Neoplasms*	*(140–239)*	*555*	*478*	*116**	*512*	*108*	*195*	*296*	*66**	*244*	*80**
Malignant	(140–205)	546	469	117*	503	109	190	278	68*	236	81*
Benign	(210–239)	9	9	100	9	100	5	18	28*	8	(†)
III Allergy, endocrine, etc	(240–289)	51	69	74‡	68	75*	17	66	26*	42	40*
IV Blood-forming organs	(290–299)	6	11	55	11	55	4	9	44	6	(†)
V Mental diseases	(300–326)	5	21	24*	12	42‡	3	7	(†)	3	(†)
VI Nervous and sensory	(330–398)	335	596	56*	376	89‡	137	330	42*	140	98
VII Circulatory system	(440–468)	846	1679	50*	1562	54*	210	742	28*	383	55*
VIII Respiratory system	(470–527)	112	267	42*	140	80‡	36	139	26*	47	77
IX Digestive system	(530–587)	115	163	71*	137	84	28	85	33*	51	55*
X Genitourinary	(590–637)	58	240	24*	100	58*	26	100	26*	29	90
XI Pregnancy and puerperium	(640–689)	—	—	—	—	—	11	41	27*	9	122
XII Skin and cellular tissue	(690–716)	4	5	(†)	2	(†)	2	4	(†)	2	(†)
XIII Bones and organs of move	(720–749)	2	8	(†)	6	(†)	5	5	(†)	3	(†)
XIV Congenital malformation	(750–759)	39	30	130	37	105	30	23	130	31	97
XV Early infancy	(760–776)	90	193	47*	120	75*	76	147	52*	84	90
XVI Senility and ill-defined	(780–795)	19	217	9*	38	50*	2	96	2*	12	17*
XVII Violence	(800–999)	278	531	52*	339	82*	77	139	55*	81	95
All causes	(001–999)	2, 752	4, 875	56*	3, 572	77*	916	2, 411	38*	1, 207	76*

*Significantly different from 100 at $P = 0.014$ or less.
†No SMR computed where both expected and recorded deaths are less than 9.
‡Significantly different from 100 at $P = 0.015$ to 0.054.

the cancer experience of the 2 groups was sufficiently similar to warrant combining them. The results of analyses directed toward this end are not presented. They showed, however, that mortality for all cancer was 13 percent higher among Japanese residing in Hawaii, a difference of borderline statistical significance ($P = 0.03$). The only individual sites exhibiting much apparent difference were cancer of the stomach, for which the rate was 22 percent higher ($P = 0.04$), and cancer of the esophagus, for which the rate was 44 percent higher ($P = 0.10$) among male Japanese in Hawaii. With these sites excluded, there was no difference in cancer mortality between the 2 groups of American Japanese.

With the reservation that mortality from cancer of the stomach and cancer of the esophagus might be slightly higher in Hawaiian Japanese than in Japanese in the United States, these comparisons developed no valid reasons for not combining the data. Subsequent analyses, therefore, compare United States white and nonwhite experience with that of Japanese residents of Hawaii and the Continental United States combined.

Observed and Expected Mortality Attributed to Cancer

The differences in cancer mortality between the Japanese and the white or the nonwhite, which appear to be of some interest, are illustrated in text-figure 1 and the detailed data from which this chart is derived are given in table 2. It is immediately evident in text-figure 1 that, when compared to either the white or the nonwhite experience, mortality attributed to all cancer is significantly excessive among Japanese males and significantly lower among Japanese females.

Among Japanese males the excess over nonwhite male experience is largely contributed by excesses of cancer of the esophagus, stomach, liver, pancreas, and lung and to lymphosarcoma, all of these differences being of reasonably great magnitude and, in addition, statistically significant. When compared to white male experience, the Japanese ratios for the same sites are all numerically greater than 100, but the excess is statistically significant only for cancer of the esophagus, stomach, and liver. It is noted, further, that all of these 6 sites are also numerically excessive among Japanese females, though only the ratios for cancer of the stomach and primary cancer of the liver are significantly greater than both the white and nonwhite ratios.

It is thus clear that during this period both male and female Japanese residents of Hawaii and the United States experienced substantially greater mortality attributed to both cancer of the stomach and primary cancer of the liver than either the white or nonwhite United States populations. In addition, mortality attributed to esophageal cancer was significantly excessive in Japanese males compared to both white and nonwhite rates and in females compared to white rates. Cancers of the pancreas and lung and lymphosarcoma were all numerically excessive among the Japanese but the differences were not statistically significant for all sex and race comparisons.

TEXT-FIGURE 1.—Malignant neoplasms, selected sites: SMR's for Japanese of the United States and Hawaii, 1949–52, and deaths expected among them on the basis of United States white and United States nonwhite rates, 1950–51. SMR's are not shown if both expected and recorded deaths are fewer than 9.

TABLE 2.—*Cancer deaths recorded among the Japanese of Hawaii and the United States during 1949–52 and expected deaths for the same period on the basis of age-specific rates for 1950 and 1951 among United States white and nonwhite males and females, and resultant standardized mortality ratios (SMR's)*

Cancer site*	International list number	Males					Females				
		Number of male recorded deaths	Basis of U. S. nonwhite rates		Basis of U. S. white rates		Number of female recorded deaths	Basis of U. S. nonwhite rates		Basis of U. S. white rates	
			No. of expected deaths	SMR	No. of expected deaths	SMR		No. of expected deaths	SMR	No. of expected deaths	SMR
All cancer	(140–205)	1,072	870.4	123†	939.3	114†	435	623.4	70†	546.6	80†
Lip, tongue, oral cavity, and pharynx	*(140–148)*	*29*	*26.9*	*108*	*34.0*	*85*	*3*	*7.5*	‡	*5.1*	‡
Digestive tract	*(150–156A, 157–159)*	*698*	*402.3*	*174†*	*388.9*	*179†*	*233*	*182.9*	*127†*	*175.3*	*133†*
Esophagus	(150)	67	40.6	165†	24.7	271†	9	7.3	123	3.7	243†
Stomach	(151)	395	173.9	227†	128.3	308†	125	57.8	216†	39.4	317†
Intestines and rectum	*(152–154)*	*104*	*96.5*	*108*	*145.5*	*71†*	*35*	*69.2*	*51†*	*84.1*	*42†*
Intestines	(152–153)	63	59.0	107	94.1	67†	21	47.0	45†	62.5	34†
Rectum	(154)	41	37.5	109	51.4	80	14	22.2	63	21.6	65
Liver, primary, or not stated, and biliary passages	*(155–156A)*	*64*	*43.8*	*146†*	*37.1*	*173†*	*36*	*24.7*	*146§*	*26.1*	*138§*
Liver and biliary passages, primary	(155)	44	15.8	278†	15.8	279†	24	8.8	273†	14.6	164†
Liver, unstated whether primary or secondary	(156A)	20	28.0	71	21.3	94	12	15.9	75	11.5	109
Pancreas	(157)	59	37.5	157†	46.9	126	25	17.1	146	18.2	137
Peritoneum, other, and unspecified	(158–159)	9	10.1	89	6.4	‡	3	6.8	‡	3.8	‡
Respiratory system	*(160–164)*	*153*	*116.3*	*132†*	*150.2*	*102*	*23*	*20.5*	*112*	*20.6*	*112*
Trachea, bronchus, and lung	(162–163)	139	98.9	140†	130.5	107	19	17.3	110	17.9	106
Other	(160, 161, 164)	14	17.4	80	19.7	71	4	3.2	‡	2.7	‡

Breast and genitourinary organs	*(170–181)*	*59*	*180. 1*	*33†*	*169. 8*	*35†*	*101*	*317. 6*	*32†*	*236. 5*	*43†*
Breast	(170)	—	2. 0	‡	2. 0	‡	24	90. 6	26†	102. 8	23†
Genital organs	*(171–179)*	*30*	*138. 2*	*22†*	*109. 2*	*27†*	*71*	*208. 6*	*34†*	*116. 9*	*61†*
Uterus	*(171–174)*	—	—	—	—	—	*54*	*180. 3*	*30†*	*79. 7*	*68†*
Cervix	(171)	—	—	—	—	—	38	101. 0	38†	43. 4	88
Other and unspecified uterus	(172–174)	—	—	—	—	—	16	79. 3	20†	36. 3	44†
Ovary, fallopian tube, and broad ligament	(175)	—	—	—	—	—	16	23. 4	68	34. 1	47†
Other and unspecified female genital organs	(176)	—	—	—	—	—	1	4. 9	‡	3. 1	‡
Prostate	(177)	24	130. 9	18†	100. 1	24†	—	—	—	—	—
Testis, other, and unspecified male	(178–179)	6	7. 3	‡	9. 1	66	—	—	—	—	—
Kidney, bladder, and other urinary	(180–181)	29	39. 9	73	58. 6	49†	6	18. 4	33†	16. 8	36†
Other and unspecified sites	*(156B, 165, 190–199)*	*65*	*86. 3*	*75§*	*105. 2*	*62†*	*38*	*63. 0*	*60†*	*62. 3*	*61†*
Skin	(190–191)	5	6. 4	‡	17. 4	29†	4	4. 5	‡	7. 4	‡
Brain and other parts of nervous system	(193)	12	9. 6	125	22. 3	54§	12	6. 9	174§	13. 4	90
Bone and connective tissue	(196–197)	12	11. 6	103	13. 9	86	4	7. 1	‡	6. 7	‡
Other	(156B, 165, 192, 194, 195, 198–199)	36	58. 7	61†	51. 6	70§	18	44. 5	40†	34. 8	52†
Leukemia and lymphoma	*(200–205)*	*68*	*58. 5*	*116*	*91. 2*	*75§*	*37*	*31. 9*	*116*	*46. 8*	*79*
Lymphoma and mycosis fungoides	*(200–203, 205)*	*34*	*30. 6*	*111*	*43. 0*	*79*	*20*	*15. 7*	*127*	*20. 4*	*98*
Lymphosarcoma and reticulosarcoma	(200)	24	10. 9	220†	18. 7	128	11	5. 4	204§	8. 8	125
Hodgkin's disease	(201)	1	9. 7	10†	14. 6	7†	6	5. 2	‡	6. 8	‡
Other lymphomas and mycosis fungoides	(202, 203, 205)	9	10. 0	90	9. 7	93	3	5. 1	‡	4. 8	‡
Leukemia and aleukemia	(204)	34	27. 9	122	48. 2	71§	17	16. 2	105	26. 4	64

*Italicized figures represent totals of 2 or more listed specific cancer sites.

†Difference not significant at $P=0.014$ or less.

‡No SMR computed where both expected and recorded deaths are fewer than 9.

§Difference not significant at $P=0.015$ to 0.054.

The ratio for cancer of the intestines is materially and significantly lower in Japanese females as compared to both white and nonwhite experience. This is also true for Japanese males as compared to the United States white rates. When compared to the nonwhite experience, however, the Japanese male ratio is numerically, though not significantly, greater.

Among Japanese males the ratios for Hodgkin's disease are significantly low.

Deaths attributed to cancers of the kidney, bladder, and other parts of the urinary tract were materially fewer among both Japanese males and females. These differences are also significant in all instances except for Japanese males as compared with nonwhite experience.

For cancer of the skin it is interesting to note that, although few deaths were expected among the Japanese, the observed numbers were considerably smaller for males than would be expected if white male mortality rates had prevailed, 5 observed and 17.4 expected, and somewhat smaller for females than if white female rates had obtained, 4 compared to 7.4 expected. However, no appreciable difference is noted when nonwhite rates of either sex are compared.

There is a numerical, but not significant, deficit of cancer of the brain and other parts of the nervous system and of the leukemias among both male and female Japanese on the basis of comparison with the white race. The reverse, however, is true when comparisons are made with the nonwhite population.

For the reproductive sites, mortality attributed to cancer of the prostate is materially and significantly less than observed in either the white or nonwhite. The deficit in cancer of the breast among female Japanese is similarly substantial. The deficit in cancer of the uterus is less than that for cancer of the breast, but is impressive nevertheless. The uterine-cancer deficit is due largely to "unspecified parts of the uterus" or to cancer of the body of the uterus, since the deficit of cervical cancer as compared with the white experience is not statistically significant.

Cancers of the ovary, fallopian tubes, and broad ligaments are significantly low on the basis of the white experience, but the deficit in this group is not significant as compared to the nonwhite experience.

It is interesting to note that in the residual group of malignancies, which include the few remaining and unspecified sites, there is a significant deficit of recorded deaths for Japanese males and females compared to those expected if either nonwhite or white rates prevailed. Thus, as was true for deaths due to ill-defined causes or senility, no appreciable allowance has to be made for any effect of redistributing these deaths if the true causes were known.

Cancer Mortality in Japan

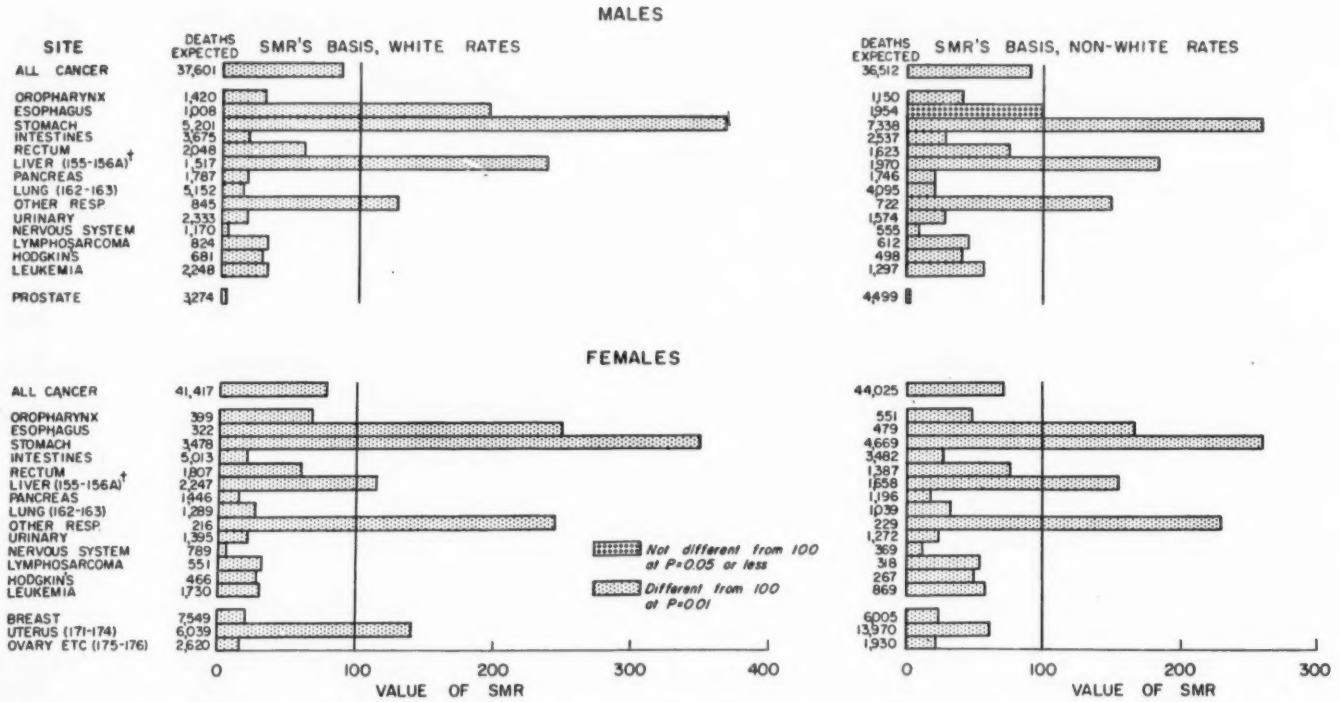
Segi (7) has compared cancer death rates in Japan for 1950 with rates recorded in England and Wales (1950) and with the rates for the white and nonwhite populations of the United States (1949) by means of total

site-specific rates age-adjusted to the population of Japan in 1950. Text-figure 2 was prepared from data presented by Segi summarizing his findings (table 10 of his report). The figure shows interesting similarities with, and differences from, the data of text-figure 1. For cancer of all sites the rate in Japan was only 87 percent of the white male and 89 percent of the nonwhite United States male rates, and 77 percent and 72 percent, respectively, for females. This is in sharp contrast with the excessive total-cancer mortality (text-fig. 1) among Japanese males residing in the United States and Hawaii. For the individual sites there were only 5 which were excessive for Japan in 1 or more comparisons. Cancers of the stomach and parts of the respiratory system other than lung were higher in Japan than among either white or nonwhite males and females. This was also true for cancer of the liver, but less reliance can be placed on the observed difference because the data for Japan include an unknown number of secondary liver cancers (156B). Cancer of the esophagus was excessive in Japan except when compared to the United States nonwhite male rates. Cancer of the uterus was excessive in Japan when compared to United States white rates. Rates for cancer of the pancreas and lung and for lymphosarcoma, all numerically greater among the Japanese in the United States and Hawaii, were only from 14 to 53 percent of the white and nonwhite rates among residents of Japan. For all other sites considered in text-figure 1, the difference between Segi's comparisons of Japanese and United States rates, and those comparisons shown here, were, in general, differences of degree rather than of direction.

These differences prompted more direct comparisons of mortality attributed to several cancer sites between Japanese residing in the United States and Hawaii and those in Japan. Table 3 and text-figure 3 show these comparisons, made possible by the detail provided by Segi (*6,7*) and supplemental data published by the World Health Organization (*11*). From these data it is seen that mortality due to all cancer was 32 percent higher among the Japanese males of the United States and Hawaii, while no significant difference existed for the females. However, for both sexes there was excess mortality for most of the sites. The sites for which the Japanese of the United States and Hawaii experienced significantly lower mortality were stomach, for both sexes, liver and respiratory system other than lung for males, and uterus. For females no significant difference was found for cancer of the esophagus, rectum, liver, and breast. It is of interest to note that mortality rates for cancer of the prostate, pancreas, lung, brain and other parts of the nervous system, and lymphosarcoma were all very much higher among the Japanese of the United States and Hawaii than among residents of Japan.

Discussion

The above differences noted between rates of Japan and those experienced by the Japanese of the United States and Hawaii suggest that environmental factors, as well as postulated inherent racial differences, may play a role in bringing about observed excessive cancer mortality



TEXT-FIGURE 2.—Malignant neoplasms, selected sites: SMR's for residents of Japan, 1950, and deaths expected among them on the basis of United States white and United States nonwhite rates, 1949. Calculated from data published by Segi (7). †=In Japan includes 156B, cancer of liver stated to be secondary site.

TABLE 3.—*Observed deaths for selected cancer sites recorded among the Japanese of the United States and Hawaii during 1949–52, deaths expected to occur among them on the basis of 1951 or 1952 age- and sex-specific death rates* of Japan, and resultant standardized mortality ratios (SMR's)*

Cancer site	International list number	Males			Females		
		Deaths recorded	Deaths expected	SMR	Deaths recorded	Deaths expected	SMR
All cancer	(140–205)	1, 072	812. 4	132†	435	450. 8	96
Buccal cavity and pharynx	(140–148)	29	10. 0	290†	3	3. 2	‡
Esophagus*	(150)	67	52. 6	127§	9	10. 8	83
Stomach	(151)	395	481. 2	82†	125	169. 9	74†
Intestines*	(152–153)	63	17. 0	371†	21	12. 1	174§
Rectum*	(154)	41	30. 0	137§	14	15. 4	91
Liver and biliary passages‖	(155–156A)	64	93. 4	69†	36	40. 0	90
Pancreas*	(157)	59	7. 8	756†	25	4. 0	625†
Lung and trachea	(162–163)	139	19. 8	702†	19	5. 3	358†
Other respiratory*	(160, 161, 164)	14	23. 3	60§	4	6. 3	‡
Breast	(170)	0	0. 5	‡	24	19. 8	121
Uterus	(171–174)	—	—	—	54	118. 2	46†
Ovary and other female genital	(175–176)	—	—	—	17	6. 0	283†
Prostate*	(177)	24	4. 2	571†	—	—	—
Testis and other male genital*	(178–179)	6	2. 9	‡	—	—	—
Urinary organs	(180–181)	29	11. 9	244†	6	4. 0	‡
Skin	(190–191)	5	5. 7	‡	4	3. 1	‡
Brain and other nervous system	(193)	12	0. 8	1, 500†	12	0. 5	2, 400†
Lymphosarcoma*	(200)	24	5. 4	444†	11	2. 2	500†
Hodgkin's disease	(201)	1	5. 5	‡	6	2. 3	‡
Leukemia	(204)	34	12. 7	268†	17	7. 9	215†

*Expected deaths based on death rates in Japan for 1952, all others on the 1951 rate.

†Significantly different from 100 at $P=0.014$ or less.

‡No SMR computed where both expected and recorded deaths are fewer than 9.

§Significantly different from 100 at $P=0.015$ to 0.054.

‖Expected mortality based on 1951 death rates for Japan, which include 156B (stated to be secondary).

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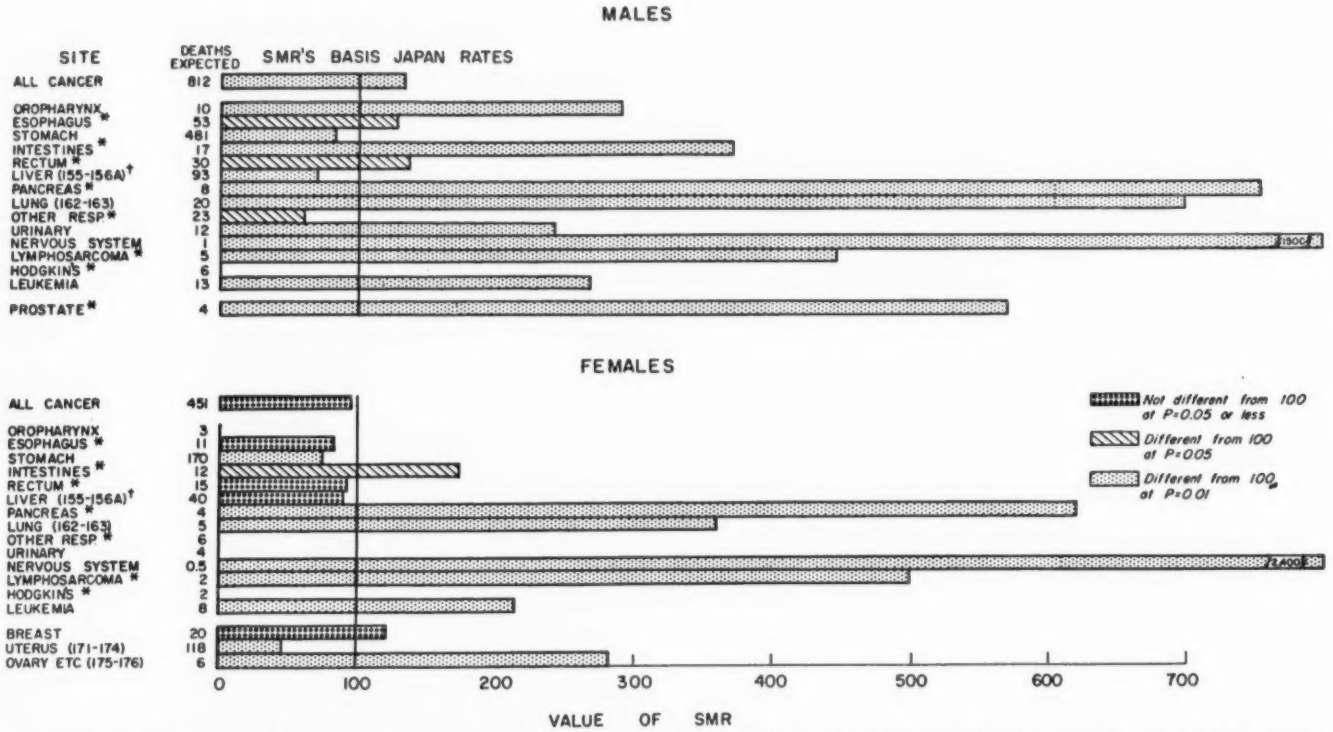

TEXT-FIGURE 3.—Malignant neoplasms, selected sites: SMR's for Japanese of the United States and Hawaii, 1949–52, and deaths expected among them on the basis of rates prevailing among Japanese in Japan during 1951 or 1952. (Sites marked with an asterisk are based on Japanese 1952 rates; all others, 1951. SMR'snot shown if both expected and recorded deaths are fewer than 9. †=In Japan includes 156B, cancer of the liver stated to be secondary.)

of some sites and deficits in others. As indicated above, there are weaknesses in comparing mortality of Japan with that of the United States for various reasons but principally because of differences in medical and death-certification practices. However, the possibility that environment may be a factor is strengthened by the fact that Japanese males of Hawaii had a higher death rate from cancer of the stomach than those residing in the United States, though the difference is of borderline significance. Further support is added when the rates of the 2 are adjusted separately to the rates of Japan. By the direct method of age adjustment it was revealed that the death rate attributed to malignancies of the stomach among the United States Japanese males was 72 percent of the 1951 mortality among males of Japan ($P=0.0001$), while the corresponding comparison for Hawaiian Japanese males was 90 percent ($P=0.35$). Among United States Japanese females the adjusted mortality for cancer of this site was 70 percent of the 1951 death rate for females of Japan ($P=0.002$). The adjusted mortality for cancer of the stomach among female Japanese of Hawaii was 75 percent of the rate for females of Japan ($P=0.014$). Nearly identical results were obtained when the indirect method of age adjustment was employed as a check.

Another way of testing the postulated effect of environment is to compare mortality among native-born and foreign-born Japanese. Unfortunately, data on mortality coded for the country of birth were available for both 1950 and 1951 only for the Japanese of Hawaii and comparison was of limited value due to the small number of deaths. Furthermore, as noted by Rhea (*8*), the native-born comprise practically all of the younger age groups and all of the older Japanese are foreign-born with appreciable overlapping only in those 45 to 54 years of age. However, on analysis it was shown that fewer native-born males died from cancer of the stomach than expected had the 1951 death rate for Japan prevailed, 6 observed compared to 15 expected ($P=0.02$). Also, fewer foreign-born males died than expected, 91 compared to 99, but the difference is proportionately less ($P=0.4$). Among females some difference was observed, 10 deaths being expected in the native-born compared to 5 observed ($P=0.1$) and 36 expected in the foreign-born as against 28 observed ($P=0.2$). These data are suggestive of a possible environmental role. On the other hand, if the same argument held, one would expect the Japanese males of the United States to have a higher mortality due to cancer of the stomach than Japanese males of Hawaii, rather than a lower death rate. Of the United States Japanese males, 30 percent are foreign-born, compared to 18 percent in Hawaii (*12*). As is the case in Hawaii, virtually all of the older Japanese of the United States are foreign-born and the younger group is native-born. Perhaps foreign-born Japanese of the United States are more prone to adopt Western customs than those of Hawaii, thus partially neutralizing the postulated effect of environment.

In this presentation we are concerned primarily with comparisons of cancer mortality among the Japanese of the United States and Hawaii with that of the white and nonwhite population of the United States.

Japanese 1952 rates; all others, 1951. SMR's not shown if both expected and recorded deaths are ... cancer of the liver stated to be secondary.)

The observation that adjusted cancer mortality rates for many sites differ markedly—and especially that some are excessive among the Japanese—adds weight to the hypothesis that the differences are real.

However, the low age-adjusted mortality for all causes among the Japanese of the United States requires further comment. The age- and sex-specific death rates occurring among them are lower in all comparisons except a few of the white age- and sex-specific rates. The deficit of mortality from all causes among the Japanese of the United States may suggest inaccuracies in the enumerated population, underrecording of deaths, or both. However, the proportion of the United States Japanese population comprising each age group differs markedly from that of the other 3 populations, principally because of the pattern of Japanese migration into the United States. The earlier immigrants were predominantly adult males. Smaller numbers of adult females immigrated some years later. Then virtually no immigration occurred for many years. The proportion of older Japanese males in the United States is higher than that of the whites or nonwhites or in the population of Japan. The unequal weighting resulting from such disproportion in the age distribution of the population, together with observed differences in age- and sex-specific mortality from all causes, may account to some extent for the over-all differences. The low mortality among the Japanese of the United States is also consistent with the idea that they are subject to forces of mortality different from those operating among other population groups.

Summary

This study compares mortality for specific cancer sites occurring among the Japanese of the United States and Hawaii from 1949 to 1952, inclusive, with that expected to occur among them had they experienced the 1950 and 1951 average annual age- and sex-specific death rates of the white or nonwhite United States population. Also included was a comparison with cancer mortality of Japan.

Cancer-mortality data for the Japanese of Hawaii and of the United States were combined after it was noted that no significant differences existed between the 2, with the exception of malignancies of the stomach and possibly esophagus, which were somewhat more frequent among Japanese males of Hawaii. Japanese males have a higher over-all cancer death rate than whites or nonwhites while the opposite is true for Japanese females. Both sexes have higher death rates than either whites or nonwhites for cancer of the esophagus, stomach, and liver and biliary passages. On the other hand, the Japanese mortality from malignancies of the intestines, including rectum, is lower except in the comparison of Japanese male mortality to nonwhite male rates. As a cause of death, malignant neoplasms of the breast, uterus, ovary, prostate, and urinary organs are less important among Japanese than among whites or nonwhites. Japanese males have fewer deaths from skin cancer than expected if white rates prevailed.

Comparison of United States white and nonwhite rates with those of Japan, previously reported by Segi (7), are summarized here and demonstrate many interesting similarities with and differences from comparisons of United States with Hawaiian Japanese. Some limitations of comparisons of mortality recorded in 1 country with that recorded in another are discussed.

The cancer mortality among the Japanese of the United States and Hawaii is demonstrated to differ significantly for many sites from that of Japan. Interesting differences are lower rates for cancer of the stomach and uterus among the Japanese of the United States and Hawaii while cancer of the lung and prostate are higher.

Foreign-born Japanese of Hawaii were shown to have somewhat higher mortality for cancer of the stomach than the native-born, but the excess was of borderline significance due to the small number of deaths and differences in age distribution of the 2 groups. The possible relationship of these differences to environmental factors is discussed.

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Leukemia Mortality. Geographic Distribution in the United States for 1949–1951 [1]

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In 1952 Clemmesen, Busk, and Nielsen (*1*) published their study of the topographic distribution of leukemia and Hodgkin's disease in Denmark, finding no certain differences. More recently Hewitt (*2*) has analyzed the regional variation of leukemia mortality in England and Wales and found a fairly regular gradient from a low level in the North to a high level in the South. Since recorded mortality from all forms of leukemia and aleukemia according to state of residence is available from the National Office of Vital Statistics, it is also possible to analyze regional variation within the United States.

The mortality attributed to leukemia and aleukemia in each state and the United States during the 3-year period 1949–51 is used in this report. Death rates for the United States as a whole, according to age, race, and sex, have been applied to the appropriate populations of individual states to determine the deaths expected in each if no regional variation from this standard United States experience existed. Dividing the recorded deaths by the expected deaths gives a standardized mortality ratio (SMR) which, when multiplied by 100, expresses the actual adjusted mortality experience in a state as a percentage of the average United States mortality.

The results of this analysis for white and for nonwhite persons, according to state of residence, are shown in table 1. The standardized mortality ratios for all persons are shown in text-figure 1. Mortality from leukemia tends to be higher in Northern United States compared to the relatively low level observed in the South and especially in the Southeast. This North to South trend is in the same direction for men and women (although not shown) of both white and nonwhite races. Regional mortality ratios have been obtained by summing the recorded and expected deaths of individual states within each of the 9 regional areas. Here, too, the same relatively high mortality is seen in the North as opposed to the low level in the South. Thus, mortality in the West North Central and Middle Atlantic regions is significantly [3] higher, and in the South Atlantic

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] A difference is considered significant herein at a level of $P=0.05$ or less.

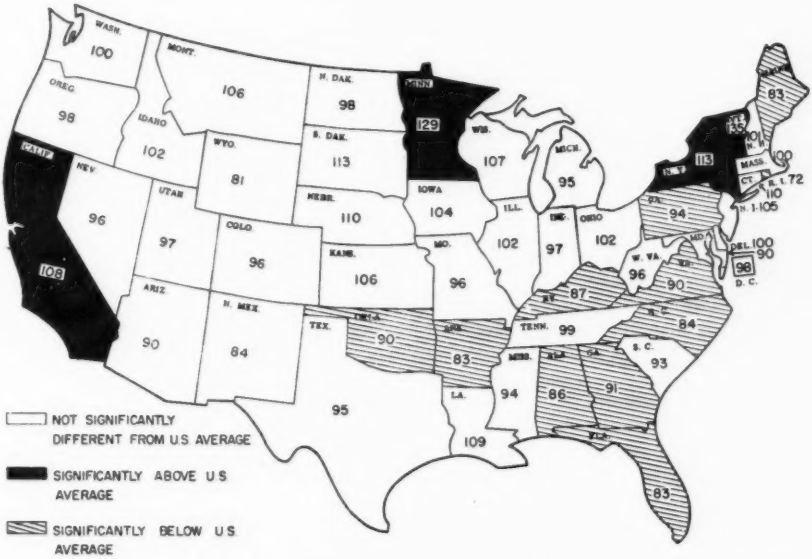
TABLE 1.—*Leukemia: Standardized mortality ratios for the states and regions of the United States by race, 1949–51*

Region and State	Number deaths—white		Standardized mortality ratio (SMR)*	Number deaths—nonwhite		Standardized mortality ratio (SMR)*
	Observed	Expected		Observed	Expected	
New England	*1,766*	*1,783*	*99*	*21*	*15*	*140*
Maine	148	180	82†	1	0	—
New Hampshire	109	108	101	0	0	—
Vermont	101	75	135‡	0	0	—
Massachusetts	900	905	99	10	8	125
Rhode Island	103	145	71‡	3	2	—
Connecticut	405	370	109	7	5	140
Middle Atlantic	*5,553*	*5,317*	*104‡*	*224*	*178*	*126‡*
New York	2,982	2,644	113‡	102	87	117
New Jersey	876	841	104	35	30	117
Pennsylvania	1,695	1,832	93‡	87	61	143‡
South Atlantic	*2,449*	*2,704*	*91‡*	*373*	*453*	*82‡*
Delaware	52	51	102	4	5	—
Maryland	296	339	87†	42	35	120
District of Columbia	97	95	102	20	25	80
Virginia	382	425	90†	61	68	90
West Virginia	300	320	94	17	11	155
North Carolina	391	470	83‡	83	91	91
South Carolina	202	201	100	48	69	70†
Georgia	376	389	97	63	94	67‡
Florida	353	414	85‡	35	55	64‡
East North Central	*5,369*	*5,387*	*100*	*208*	*172*	*121‡*
Ohio	1,431	1,417	101	58	49	118
Indiana	692	717	97	17	17	100
Illinois	1,557	1,541	101	76	63	121
Michigan	991	1,060	93†	54	40	135†
Wisconsin	698	652	107	3	3	—
West North Central	*2,893*	*2,676*	*108‡*	*58*	*49*	*118‡*
Minnesota	731	569	128‡	8	3	267‡
Iowa	544	522	104	2	2	—
Missouri	706	735	96	33	31	106
North Dakota	109	110	99	0	1	—
South Dakota	137	119	115	0	2	—
Nebraska	280	258	109	6	2	300‡
Kansas	386	363	106	9	8	112
East South Central	*1,375*	*1,494*	*92‡*	*220*	*251*	*88*
Kentucky	419	484	87‡	21	22	95
Tennessee	458	467	98	57	52	110
Alabama	297	340	87†	69	88	78†
Mississippi	201	203	99	73	89	82
West South Central	*1,940*	*2,054*	*94‡*	*240*	*238*	*101*
Arkansas	226	264	86†	27	42	64†
Louisiana	301	287	105	102	82	124†
Oklahoma	342	376	91	16	20	80
Texas	1,071	1,127	95	95	94	101
Mountain	*800*	*842*	*95*	*21*	*20*	*105*
Montana	117	109	107	1	2	—
Idaho	105	103	102	0	0	—
Wyoming	35	48	73	4	0	—
Colorado	230	240	96	4	3	—
New Mexico	84	97	87	2	5	—
Arizona	97	108	90	7	8	88
Utah	106	110	96	2	1	—
Nevada	26	27	96	1	1	—
Pacific	*2,710*	*2,574*	*105†*	*84*	*71*	*118*
Washington	440	440	100	8	6	133
Oregon	279	285	98	4	3	—
California	1,991	1,849	108‡	72	62	116
Total	24,855	24,831	100	1,449	1,447	100

*SMR's not calculated where observed and expected deaths are under 5. †P=0.02 to 0.05. ‡P=0.01 or less.

region lower, for both white and nonwhite persons. Other significant variations include an excess mortality in the Pacific region and a deficit in both the East and West South Central regions for white persons, and for nonwhites an excess in the East North Central region. There are no regions in which white and nonwhite mortality rates differ significantly in opposite directions. With 2 exceptions this is also true for individual states. These exceptions are Pennsylvania and Michigan, in which mortality among white persons is significantly lower, and among nonwhites higher, than the average United States experience for each group.

STANDARDIZED MORTALITY RATIOS FOR LEUKEMIA AMONG THE STATES OF THE UNITED STATES, 1949-51



TEXT-FIGURE 1.—Standardized mortality ratios for leukemia among states of the United States, all persons, 1949–51 (significance where shown is at level of $P = 0.05$ or less).

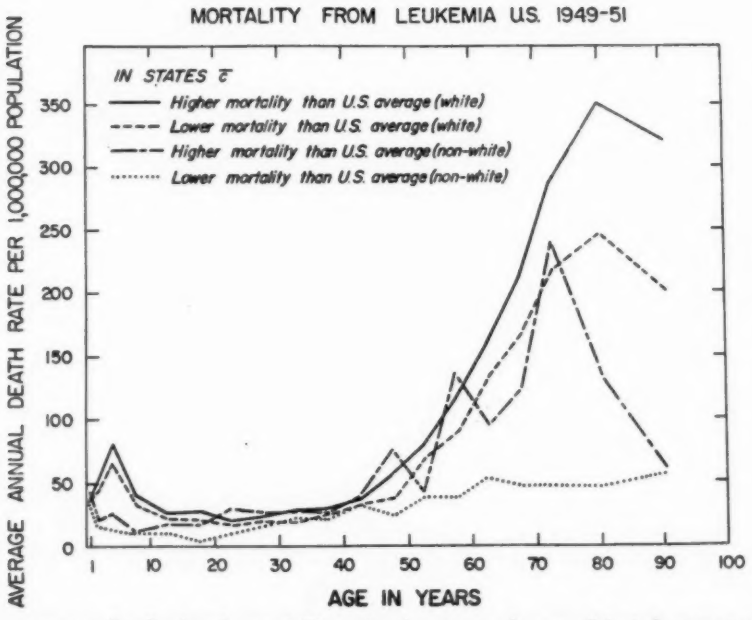
In order to determine whether or not geographic variation is more pronounced in 1 age group than in another, the recorded and expected deaths of the high-mortality states and the low-mortality states (as shown in text-fig. 1) have been used to obtain mortality ratios for 6 age groups (table 2). In states with high leukemia mortality rates, the proportionate excess over corresponding rates for the United States is greatest, 22 percent, in the age group 5 to 24 years and smallest, 8 percent, from 0 to 4 years of age. It increases gradually from 10 to 16 percent at ages over 24 years. In states with low mortality rates the deficit tends to increase with age, although it is slightly less in the oldest age group than at 65 to 74 years. The relative difference between the excess mortality in high-rate states and the deficit in mortality in low-rate states is smallest at

ages 0 to 4 years and greatest in the oldest age group, where it is twice that in the youngest age group. Therefore, although the regional variation is present at all ages, it increases with age and tends to be greatest in the oldest age groups. This variation with age can be seen by plotting age-specific death rates (text-fig. 2), and is a characteristic of both white and nonwhite mortality curves.

TABLE 2.—*Mortality ratios for several age groups in 4 states with significantly high mortality rates and in 11 states with significantly low mortality rates**

Age	High-rate states	Low-rate states	Total United States
0–4	108	93	100
5–24	122	93	100
25–44	110	92	100
45–64	113	90	100
65–74	111	81	100
75+	116	84	100

*$P = 0.05$ or less.



TEXT-FIGURE 2.—Leukemia mortality rates by age and race: Selected groups of states with significantly ($P = 0.05$ or less) high and significantly low mortality from leukemia, 1949–51.

It has long been recognized that the recorded mortality from leukemia is lower for nonwhite Americans than for white Americans. Such differences are usually attributed to the influence of social conditions, especially to inadequate diagnostic facilities. The similarity of geographic

distribution of leukemia among whites and nonwhites is certainly consistent with such reasoning. On the other hand, the age differential in rates is difficult to reconcile with the explanation that social conditions are a key factor. Thus, as pointed out by Hewitt (*2*), the total mortality among nonwhites is lower, as is mortality at each extreme of life, but it is greater than white mortality between ages 35 to 44. In this connection the mortality rates in children up to 5 years of age are of special interest. The cause of the very unusual peak between 3 to 4 years of age has been a subject of speculation for some time. Cooke (*3*) in 1942 compared it with the incidence, according to age, of acute childhood infections, which could be important etiologic factors. Hewitt has shown recently that this peak has been accentuated since 1931 and especially among the first postwar babies. Mortality curves for white children in both high- and low-mortality states have this characteristic peak. Nonwhite rates, although of the same magnitude as white rates in babies under 1 year of age, decrease with age as the white rate increases. In states with a high nonwhite mortality the rate at 3 to 4 years is greater than at 1 to 2 years, but is still less than mortality under 1 year of age.

Assuming that nonwhite children experience acute infections no less often that do white children, a causal relationship between infection and leukemia should manifest itself by a peak in nonwhite as well as white rates. On the other hand, it is extremely difficult to ascribe the low nonwhite mortality to inadequate diagnostic facilities, since they seem to be quite adequate for children under 1 year, considering the similarity in rates. If this represents a real difference in risk of death between white and nonwhite children, the reasons are not yet apparent but probably represent environmental differences. As an example, immunization procedures might be mentioned as a possibility. If immunization is received more frequently by white children and is in any way leukemogenic, such differences in mortality curves could reasonably be expected.

Discussion

These data demonstrate surprisingly little regional variation in leukemia mortality among the states of the United States. This can be seen by a comparison with regional variation in lung-cancer mortality as analyzed by Hoffman and Gilliam (*4*). Standardized mortality ratios for lung cancer ranged from 53 in the states with the lowest death rate to 140 in the state with the highest, whereas the SMR's for leukemia have a much smaller range—from 72 to 135. Meadors (*5*) has shown an excess urban leukemia mortality over rural, and if this were taken into account it would tend to raise the lower death rates in the Southern, more rural states, resulting in even less regional variation.

Summary

1) Recorded mortality from leukemia tends to be greater in the Northern United States than in the Southern United States. This is true for both males and females of white and nonwhite races.

2) States with a significantly higher-than-average death rate have excess deaths at all ages, but the excess increases with age.

3) The peak in death rate among white children between 3 and 4 years of age is not observed in nonwhite death rates.

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Responses of Seven Inbred Strains of Mice to Percutaneous Applications of 3-Methylcholanthrene [1]

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Experiments reported here were performed following requests during 1950 from numerous investigators who were interested in the response of inbred strains of mice maintained in this laboratory to percutaneous applications of carcinogenic hydrocarbons. This information was not available, and a survey of the literature at that time indicated that few investigators had been interested primarily in this problem.

Branch (*1*) applied a 0.5 percent solution of dibenz[*a*,*h*]anthracene in benzene biweekly for 15 to 53 weeks to strains C57BL and A and found the former to be more susceptible. Bonser (*2*) compared the susceptibility of 4 inbred strains and used 2 carcinogens. A 0.3 percent solution of methylcholanthrene or benzo[*a*]pyrene was painted once weekly on the skin of strains IF, A, CBA, or Kreyberg's "white label." She found that following painting with methylcholanthrene papillomas appeared earlier in the IF strain than in the CBA strain and that following applications of benzopyrene the order of susceptibility was IF, white label, CBA, and A.[3] Mider and Morton (*4*) exposed strains C57BL and C57BR to a single painting of 0.5 percent methylcholanthrene in benzene and found more papillomas in the C57BR animals. Lauridsen and Eggers (*5*), during a study of the effect of irritation on epidermal carcinogenesis, painted strains C57BL and C3H with a 0.4 percent solution of dibenz[*a*, *h*]anthracene in benzene twice weekly and found strain C57BL to be the more susceptible. Cowdry and Suntzeff (*6*) used strains CBA and New Buffalo to determine the influence of age upon epidermal carcinogenesis. Two drops of a 0.6 percent solution of methylcholanthrene in benzene were applied 3 times weekly for 3 months to male mice. Young New Buffalo mice were more susceptible than young CBA, but strain differences in susceptibility were not apparent in older mice.

Recently, Shubik and Ritchie (*7*) used males of strain C3H and females of the Swiss strain for experiments in which the animals received 1 to 3 paintings of 1.0 percent or 0.2 percent 9,10-dimethyl-1,2-benzanthracene

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] In the publication referred to, Bonser used Bagg albino mice. In a subsequent paper, however, she stated the Bagg albino mice were in reality Strong's strain A (*3*).

in mineral oil followed by repeated applications of croton oil in mineral oil. In their discussion they stated that "the C3H mice appear considerably less responsive to this form of carcinogenesis than the Swiss mice."

Wynder, Graham, and Croninger (*8*) tested cigarette tar for carcinogenic activity by painting strain C57BL and Swiss mice and concluded that the latter animals were the more susceptible. Kotin, Falk, and Thomas (*9*) detected carcinogenic hydrocarbons in the exhaust products of diesel engines and applied exhaust extracts to the skin of inbred mice. Papillomas occurred in strain C57BL, while strain A animals developed both papillomas and carcinomas. Their results suggest that strain A was more susceptible than strain C57BL. Clemo, Miller, and Pybus (*10*) also used inbred mice to test chemical fractions from industrial smoke and diesel-engine fumes for carcinogenic properties. They found that strain C57BL "seemed to be" more susceptible than strain A. These 3 papers reveal the practical importance of suitable experimental animals when testing for the presence of carcinogens.

This review covers a small fraction of the literature on epidermal carcinogenesis. The reader is referred to publications by Berenblum (*11*), McCarter (*12*), and Cowdry (*13*), which include complete reviews and comprehensive discussions of the many problems.

After reviewing the literature prior to 1950 it was decided to test the strains available in this laboratory for susceptibility to the carcinogenic hydrocarbon 3-methylcholanthrene to ascertain whether any strain showed such a high degree of susceptibility that it could be used for the detection of carcinogenic hydrocarbons. It was found that strain I mice developed papillomas much earlier than 6 other inbred strains.

Materials and Methods

Mice.—Seven strains of inbred mice were used. With the exception of strain A, all were procured from colonies maintained in this laboratory. Strain A mice were obtained from the breeding colony of the National Institutes of Health and, in accordance with accepted nomenclature (*14*), may be designated as strain A/N.

A litter of strain BALB/c was obtained from Dr. G. D. Snell in 1935 when the strain was in the F_{35} generation. Animals used in this study were from the F_{72} and F_{74} generations. Establishment of a high-mammary-tumor line of BALB/c mice from the low-mammary-tumor strain during 1940 by foster nursing upon strain C3H was recorded in earlier papers (*15,16*). Mice from the F_{33} generation of the high-tumor line were used.

Strain C3H has been propagated in this laboratory since 1933 and is an established high-mammary-tumor strain. Ancestors of the colony were supplied by Dr. L. C. Strong (*16*). Animals used in this work were from the F_{53} and F_{56} generations. An agent-free line of the strain was started in 1942 and shows a low incidence of mammary tumors (*17*). Mice used here were from the F_{22} generation of this line.

Strain C57BL animals descended from a litter procured from Dr. W. S. Murray during 1939. This colony, in common with others, is a

low-breast-tumor line (*17*). Strain C57BL mice used here were of the F_{27} and F_{28} generations in this laboratory. Strain I mice were descendants from a litter obtained during 1935 from Dr. J. M. Murray (*17*). This strain is a low-mammary-tumor strain and those used were in the F_{57} and F_{58} generations. Strain DBA/2 mice freed of the mammary tumor agent during 1942 were used (*17*). Members of the F_{14} generation of this line were available.

A colony of strain RIII mice was started with a litter supplied by Dr. W. H. Woglom during 1945. Descendants of these mice were bred brother to sister for 20 generations, and a line is available in which virtually all breeding and nonbreeding females develop mammary tumors. Animals from the F_{17} generation of breeding in this laboratory were used. An agent-free line of this strain was started during 1950 by surgical removal of a litter from the uterus of an RIII mouse and foster nursing this litter on a C57BL mouse. The first 6 generations contained 90 females, of which 3 developed mammary tumors. Mice from the F_2 and F_3 generations of the agent-free line were used.

All mice were nonbreeders, and were kept 8 to a cage in the same room throughout the experiment. A diet of Purina laboratory chow pellets and water was available at all times.

Carcinogen.—A 0.25 percent solution of methylcholanthrene (m.p. 179–180° C.) dissolved in reagent (thiophene-free) benzene (Merck) was used. It was applied, at weekly intervals, to the unshaven skin of the interscapular area by a single upward stroke of a No. 6 camel's hair brush. All applications were made by the same individual. An adequate supply of the carcinogen in benzene was prepared for each experiment and was kept in a glass-stoppered container in the dark. An amount sufficient for 1 application was removed from the container at each painting.

Tumors.—The experimental animals were examined each week for the presence of papillomas and, in order to avoid equivocal diagnoses, a lesion was not called a papilloma until it was approximately 2 mm. in diameter. A mouse in which a papilloma grew progressively was killed when the lesion had attained a large size and definite induration at the base indicated invasion of deeper tissues. A few animals became ill and were sacrificed while their lesions were small.

At autopsy the number of lesions was recorded, each was measured, and a notation was made of any peculiar gross characteristics, such as ulceration, degree of hardness, or location of a subcutaneous mass not obviously adjacent to a surface lesion. Histologic sections stained with hematoxylin and eosin were prepared from virtually all lesions. Tissues were fixed in Tellyesnicky's fluid (2 parts 40% formaldehyde solution, 20 parts 70% alcohol, and 1 part acetic acid), blocked in paraffin, cut, and stained with hematoxylin and eosin. Histologic diagnoses were not made of lesions from the small number of mice that died or the few that became ill and were killed while bearing a very small papilloma. The total number of such animals had no significant bearing on the outcome of the experiments.

The tumors were classified by microscopic examination into 4 groups: carcinomas, questionable carcinomas, papillomas, and sarcomas. The criteria employed for the diagnosis of *carcinoma* of the skin were: 1) invasion of the panniculus carnosus or subcutaneous veins and lymphatics by neoplastic epithelial cells, and 2) anaplasia of epithelial cells. *Questionable carcinomas* included tumors in sections of which epithelial anaplasia and hyperplasia were present in the absence of invasion. Some of the tumors in this class were acutely inflamed and ulcerated. *Papillomas* included tumors in microscopic sections of which epithelial hyperplasia and hyperkeratosis were seen in the absence of platysmal and vascular invasion by epithelial cells and epithelial anaplasia. *Sarcoma* included those anaplastic invasive tumors which were demonstrated by phosphotungstic acid-hematoxylin and Mallory's trichrome stains to originate from muscle or connective tissue.

Experimental Procedures and Results

Two experiments were performed. In the first, mice of strains A, BALB/c, C57BL, C3H, DBA/2, I, and RIII were used. All strain A mice and approximately half those of strains BALB/c and C3H carried the mammary tumor agent; all others were agent-free. They were between 1 and 3 months of age and each strain was divided equally between the sexes. Each mouse received 20 applications of the carcinogen. All mice lost the hair at the site of application, and C57BL mice experienced a definite loss in weight. None of the other strains showed any obvious deleterious effects of painting.

The results are summarized in table 1. Mice of each strain are combined, for there was no significant difference in response either between sexes or in mice free of, and those harboring, the mammary tumor agent. With the exception of strain C57BL all developed papillomas. Three C57BL females did not develop papillomas—1 of these died 41 weeks and 2 died 52 weeks after the first painting.

The course of development of lesions in 4 strains was similar to the description by Branch (*1*), but the lesions in strains I, C3H, and C57BL each had a characteristic appearance. Strain I animals developed lesions which were heavily keratinized and frequently gave rise to "epithelial horns" up to 25 mm. long. These structures also appeared in mice of other strains but far less frequently than in strain I—the longest arose in an RIII male and at autopsy was 30 mm. in length. Strain C3H animals had a tendency to grow "fleshy" appearing papillomas instead of the usual keratinized warts. A few C3H mice also developed subcutaneous masses beneath the painted area before a papilloma arose on the surface. Histologic examination revealed that these masses were sarcomas with an overlying carcinoma or papilloma. Strain C57BL mice reacted to the carcinogen by producing the usual wart-like lesions, but at autopsy their lesions differed from those in other strains in that they were more uniform in appearance. Virtually all were round and flat and consisted of firm white tissue.

TABLE 1.—*Response of 7 inbred strains of mice to 20 weekly paintings of 0.25 percent methylcholanthrene in benzene*

Strain	No. of mice	No. developing papilloma	No. developing more than 1 papilloma	Average time between first painting and papilloma (*weeks*)	Average time between papilloma and autopsy (*weeks*)	Histologic diagnosis					No. in which papilloma regressed	No. failing to develop papilloma	Average time between first painting and death without papilloma (*weeks*)
						No. of mice	No. with carcinoma	No. with questionable carcinoma	No. with papilloma	No. with sarcoma			
A	79	79	12	20	10	77	65	4	5	7	0	0	—
BALB/c	197	197	57	21	10	191	178	2	1	10	1	0	—
C57BL	103	100	20	22	14	95	85	2	0	12	2	3	48
C3H	200	200	64	19	9	197	181	3	2	22	1	0	—
DBA/2	82	82	27	21	14	79	78	1	0	1	0	0	—
I	97	97	57	15	12	94	90	2	0	4	0	0	—
RIII	107	107	15	24	19	91	84	2	0	7	1	0	—

Multiple papillomas arose with greater frequency in strain I than in any other strain. They often developed from 10 to 15 lesions which coalesced to form a single large papilloma, whereas mice of other strains usually developed 2, and rarely as many as 4, papillomas.

Strain I animals also developed papillomas earlier than the other strains. The average interval of 15 weeks between the first painting and the occurrence of papillomas indicates that nearly all strain I mice had grown papillomas before the last application of carcinogen. The first strain I papilloma was found 8 weeks and the last, 25 weeks after the initial painting. Only 5 (2, 1, 1, and 1 at 21, 22, 23, and 25 weeks, respectively) appeared following the last painting. Of the other strains, 48 strain A, 88 strain BALB/c, 43 strain C57BL, 105 strain C3H, 31 strain DBA/2, and 16 strain RIII had developed papillomas prior to their last painting.

The sixth column of the table shows that, on the average, mice of strain C3H were the first to come to autopsy after the appearance of papillomas. This is not presented as evidence that the C3H papillomas were the first to become malignant. It does indicate, however, that according to the gross criteria used to estimate malignancy, animals of strain I, despite their early response to the carcinogen, did not develop malignant tumors at a more rapid rate than other strains. Bonser (*2*) found that, following percutaneous application of methylcholanthrene to strains IF and CBA, warts appeared earlier in the IF strain but they required a longer time to become malignant.

Histologic examination revealed that a high incidence of malignant tumors was shown by all strains. In the table a larger number of tumors appear than mice supplying tissues, because a few mice developed more than 1 type of tumor. The great majority of tumors were carcinomas, which usually attend repeated application of a carcinogen to the skin. Sarcomas occurred in all strains but were most prevalent in strains C3H and C57BL. It is known that percutaneous application of carcinogens induces sarcomas in mice.

Only 5 papillomas regressed. Of these, only 1 (in an agent-free C3H female) arose before termination of the painting schedule. A record of the time of disappearance of these lesions was not made, but when the mice were killed 3 to 6 months after they had developed papillomas, 4 had normal-appearing skin within the painted area and 1, the C3H female, had thickened skin only, both grossly and histologically.

The results of this experiment showed clearly that the dose of carcinogen used was too large to reveal significant strain differences in response. There was definite evidence that strain I mice developed papillomas earlier and in greater numbers than other strains, but other criteria of susceptibility were similar for all strains.

The second experiment included only 5 strains because mice of strains A and DBA/2 were not available. All mice of strains C3H and RIII and half those of strain BALB/c harbored the mammary tumor agent; those of strains C57BL and I were agent-free. The mice were from 1 to 4 months

TABLE 2.—*Response of 5 inbred strains of mice to 10 weekly paintings of 0.25 percent methylcholanthrene in benzene*

Strain	Sex	No. of mice	No. developing papilloma	No. developing more than 1 papilloma	Average time between first painting and papilloma (*weeks*)	Average time between papilloma and autopsy (*weeks*)	Histologic diagnosis					No. in which papilloma regressed	No. failing to develop papilloma	Average time between first painting and death without papilloma (*weeks*)
							No. of mice	No. with carcinoma	No. with questionable carcinoma	No. with papilloma	No. with sarcoma			
BALB/c.....	F	48	40	1	27	18	38	35	1	0	2	1	8	60
BALB/c.....	M	41	38	3	25	19	37	31	1	5	1	0	3	71
C57BL.....	F	17	9	0	41	22	5	4	0	1	0	0	8	72
C57BL.....	M	11	3	0	38	4	2	1	0	0	1	0	8	72
C3H.....	F	18	4	0	17	14	4	3	0	0	1	0	14*	34
C3H.....	M	17	14	0	36	12	14	9	1	0	4	0	3	69
I.....	F	25	15	3	12	21	12	12	0	0	0	1	10	48
I.....	M	24	22	3	14	26	14	12	0	1	1	2	2	33
RIII.....	F	36	18	0	30	17	12	12	0	0	0	1	18*	35
RIII.....	M	25	24	0	29	30	19	16	1	0	3	2	1	70

*All developed mammary tumors.

old and the sex distribution for each strain was approximately equal. The dose of carcinogen was given in 10 weekly applications.

Careful records were made of the gross appearance of the painted areas and the physical condition of the animals. The majority of mice of all strains began losing hair after the first painting, and after the third painting almost all painted areas were bare and inflamed. Strain I mice showed a more pronounced reaction, for their skins were inflamed and roughened. After the fourth painting, strain C57BL mice were thin; they were the only mice to show any systemic effects from the applications of carcinogen. These conditions persisted throughout the painting period.

The results are shown in table 2. Agent-carrying and agent-free BALB/c mice are combined because their responses were similar. The sexes are not combined because most strains showed a difference in response according to sex. Four of 18 strain C3H females and 18 to 36 strain RIII females developed papillomas, but the remaining females of both strains developed mammary tumors and died without papillomas. This makes impossible any comparison between the sexes of strain C3H, but the results in strain RIII suggest that the males may have been more susceptible, since the average time of death for females without papillomas exceeded the average time at which papillomas arose in males. Males and females of strain BALB/c showed approximately the same degree of susceptibility. Females of strain C57BL showed a higher incidence of papillomas than males, whereas the males of strain I showed a higher incidence than females.

These differences between sexes are suggestive only, because of the small number of animals used in most strains, but they are of sufficient importance to separate the sexes when comparing the results of this experiment with those of the first. The incidence of papillomas according to sex were as follows:

Strain	*Females (percent)*	*Males (percent)*
BALB/c	83	93
C57BL	53	27
C3H	22	82
I	60	92
RIII	50	96

Males of the C57BL strain had the lowest incidence, while males of the 4 other strains showed similar incidences. The occurrence of mammary tumors in females of strains C3H and RIII leaves only strains BALB/c, C57BL, and I for comparison. BALB/c exhibited the highest incidence while strains C57BL and I showed similar incidences. This response of strain C57BL and strain I females is of interest, for, as seen in the table, strain I females developed papillomas much earlier than did those of strain C57BL.

The incidence of papillomas in the mice of this experiment permits but 2 conclusions. First, strain C57BL males were the most resistant. Second, when determining strain differences mice of the same sex should be used.

A corollary of the second conclusion would be that only agent-free mice should be used, especially when females only are available.

All strains developed fewer papillomas than in the first experiment, and, with the exception of strain I, all required more time to develop papillomas than when exposed to 20 applications of carcinogen. Furthermore, of 262 mice painted in this experiment only 10 had more than 1 papilloma, while in the first experiment 252 of 865 mice developed more than 1 papilloma.

The time of appearance of papillomas in this experiment was of importance, for it permits an evaluation of strain susceptibility. As in the first experiment, strain I mice developed papillomas much earlier than the other strains. The average time between the first painting and appearance of papilloma in strain C57BL mice, together with their low incidence of tumors, indicated they were the most resistant. The resistance of the C57BL strain becomes more evident when the strains are compared on the basis of the number of weeks required for 50 percent of the mice to develop papillomas after their first painting. This was as follows:

Strain	*Females* (*weeks*)	*Males* (*weeks*)
BALB/c	26	21
C57BL	59	72
C3H	—	30
I	15	12
RIII	30	27

These data, together with those included in tables 1 and 2, show that strain I was the most susceptible and strain C57BL the most resistant, while strains BALB/c, C3H, and RIII were of intermediate susceptibility.

In contrast to the findings in the first experiment fewer mice of this experiment developed papillomas prior to the last application of carcinogen—8 strain I (5 males and 3 females), 1 C57BL female, 2 C3H females, and 1 RIII male. Data in table 2 show that a schedule of 10 paintings also increased the average time between the appearance of papilloma and autopsy in all strains but papillomas regressed in only 7 mice. However, despite the increased latent period and elapsed time before autopsy, all strains revealed a high incidence of malignant tumors. Apparently 10 applications of the carcinogen were sufficient to induce lesions which proceeded to malignancy.

The results of this experiment confirmed those suggested by the first one, namely, that strain I mice were the most susceptible and strain C57BL the most resistant. The dose of carcinogen was too large to permit an accurate appraisal of the relative susceptibilities of the intermediate strains BALB/c, C3H, and RIII.

Results Attending Painting with Croton Oil

During the 29th week following the first application of carcinogen, an analysis of the findings showed that the primary objective of the experi-

ment had been achieved, *i.e.*, 5 of 52 strain C57BL mice and 37 of 53 strain I mice had developed papillomas. It was decided to utilize the remaining mice of strains BALB/c, C57BL, C3H, and I to ascertain whether application of croton oil to the painted areas would influence the occurrence of papillomas in mice free of lesions. All animals were examined carefully and only those with no gross evidence of papilloma in the painted area were used. These were divided as equally as possible into groups of litter mates of the same sex; some were given croton-oil paintings while others were kept as untreated controls. Paintings were started during the 29th week of the experiment and were repeated weekly for a total of 12 applications. Each application consisted of a 5 percent solution of croton oil in olive oil administered by the same technique used previously to apply the carcinogen.

The results are summarized in table 3. Control groups in this table are included in table 2. The average length of time between first painting and occurrence of papilloma or death without papilloma represents the number of weeks after the first application of carcinogen.

All groups painted with croton oil showed a higher incidence of papillomas than their untreated control groups, and, with the exception of the C57BL males, all painted groups also developed papillomas at an earlier mean time than did their controls. Of the mice treated with croton oil that developed papillomas, 13 BALB/c females, 4 BALB/c males, 3 C57BL females, 0 C57BL males, 7 C3H females, 8 C3H males, and 4 I females did so during the period of application. Of the untreated controls 5 BALB/c females, 7 BALB/c males, 0 C57BL females, 1 C57BL male, 0 C3H females, 4 C3H males, and 0 I females developed papillomas during the same period of time. Obviously 12 applications of croton oil were not essential to elicit papillomas in most of the methylcholanthrene-treated mice.

Discussion

Strain I mice were the most susceptible to the induction of skin tumors following applications of methylcholanthrene. In the first experiment they developed papillomas earlier than other strains. In the second experiment the males showed a high incidence of papillomas, and these arose much earlier than in either sex of other strains; the females showed an incidence of papillomas that was average in comparison with other females but they, too, developed papillomas much earlier than other females or males. The skin of strain I mice may be a suitable test object for the detection of small amounts of carcinogenic hydrocarbons.

The chief objection to strain I is the difficulty in procuring large numbers of mice. In this laboratory they breed well, but the mothers neglect their young. Consequently, it is impossible to forecast the number of animals that will be available when a given number of females are mated. One peculiarity of the strain is an occasional tendency of the females to raise all their offspring for periods of 2 to 3 months before lapsing into their customary neglect. At such times an effort is made to procure as many

TABLE 3.—*Response of inbred mice to 12 weekly percutaneous applications of 5.0 percent croton oil in olive oil, starting 19 weeks after last of 10 weekly applications of 0.25 percent methylcholanthrene (M.C.A.) in benzene*

Strain	Sex	Group	No. of mice	No. developing papilloma	Average time between first M.C.A. painting and papilloma (*weeks*)	Average time between papilloma and autopsy (*weeks*)	Histologic diagnosis					No. in which papilloma regressed	No. failing to develop papilloma	Average time between first M.C.A. painting and death without papilloma (*weeks*)
							No. of mice	No. with carcinoma	No. with questionable carcinoma	No. with papilloma	No. with sarcoma			
BALB/c.....	F	Croton oil.	16	15	35	14	14	11	0	2	1	0	1	72
BALB/c.....	F	Control...	21	13	45	16	13	11	1	0	1	0	8	61
BALB/c.....	M	Croton oil.	7	6	38	16	5	4	0	1	0	0	1	39
BALB/c.....	M	Control...	15	12	41	16	11	9	0	2	0	0	3	71
C57BL.....	F	Croton oil.	13	9	44	21	5	5	0	0	1	3	4	69
C57BL.....	F	Control...	13	5	58	15	2	2	0	0	0	0	8	72
C57BL.....	M	Croton oil.	11	5	49	20	3	3	0	0	0	0	6	68
C57BL.....	M	Control...	10	2	45	5	1	1	0	0	0	0	8	72
C3H.....	F	Croton oil.	8	7*	36	6	6	2	0	1	4	0	1†	45
C3H.....	F	Control...	8	0	—	—	—	—	—	—	—	—	8‡	40
C3H.....	M	Croton oil.	11	11	37	12	11	9	0	0	2	0	0	—
C3H.....	M	Control...	11	8	44	10	8	5	0	0	3	0	3	69
I.....	F	Croton oil.	4	4	34	17	4	4	0	0	0	0	0	—
I.....	F	Control...	8	0	—	—	—	—	—	—	—	—	8	56

*Four mice also developed mammary tumors.
†Developed mammary tumor.
‡All developed mammary tumors.

mice as possible if they are needed. On a few occasions, when a considerable number were needed, newborn strain I young were foster-nursed by BALB/c females, which are excellent mothers. None of the mice used in experiments reported here were fostered.

It is of interest that, despite the susceptibility of strain I to the induction of skin tumors with methylcholanthrene, their subcutaneous tissues are relatively resistant to the carcinogen (*18*). Bonser (*3*) found the same relative susceptibility in the IF strain. This again confirms the well-known fact that within an inbred strain susceptibility to a carcinogen is more pronounced in certain tissues or organs.

Inbred strains C57BR, IF, and Swiss mice could also be satisfactory experimental animals for detection of small quantities of carcinogenic hydrocarbons. Mider and Morton showed that a single percutaneous application of a 0.5 percent solution of methylcholanthrene in benzene elicited papillomas in strain C57BR (*19*) and in strain C57BL (*4*). Those occurring in C57BL mice regressed, but a few of those in C57BR progressed to malignancy. Law (*20*) found that the more potent carcinogen 9,10-dimethyl-1,2-benzanthracene, when applied once to the skin of strain C57BR mice as a 0.3 percent solution in benzene, induced papillomas, of which approximately 50 percent became transformed to carcinomas. Strain IF is suggested because Bonser (*2*) found these mice very susceptible to the development of papillomas following applications of methylcholanthrene or benzo[*a*]pyrene.

That Swiss mice are susceptible was discovered by Cramer and Stowell (*21*), who observed papillomas and carcinomas in them after a single application of 0.3 mg. of methylcholanthrene in benzene. Shubik and Ritchie (*7*) gave 1 application of a 0.2 percent solution of 9,10-dimethyl-1,2-benzanthracene in mineral oil to Swiss mice and 5 weeks later started biweekly treatments of the painted area with croton oil. Fifty-three of 87 mice developed skin tumors. Klein (*22*) recently reported the appearance of skin tumors in 5 of 22 Swiss mice that had received a single application of a 0.007 percent concentration of 9,10-dimethyl-1,2-benzanthracene in benzene. This finding demonstrates a high degree of susceptibility for these mice.

The availability of strains of mice highly susceptible to epidermal tumors and progress in studies of dose-response relationship (*23,24*) make possible an approach to the problem of the detection of small amounts of carcinogenic hydrocarbons in substances suspected of possessing carcinogenic properties.

Strain C57BL was the most resistant to percutaneous administration of methylcholanthrene. This observation was not in accord with those of Branch (*1*), who compared strains C57BL and A, or of Lauridsen and Eggers (*5*), who used strains C57BL and C3H.

Two explanations for this discrepancy are apparent. First, strain C57BL includes several distinct substrains and all may not be of equal susceptibility. Our colony of C57BL mice descended from a litter procured in 1939, before substrain differences were recognized. Hence the rela-

tionship of these mice to those used by others, especially earlier investigators, is not known and could easily account for the different findings.

The second possible explanation could lie in the reaction of the C57BL animals of the present study to skin painting. They were the only strain to exhibit any obvious deleterious effects from application of the benzene solution of carcinogen. The animals were not weighed, but, with the exception of strain C57BL, all strains appeared to be in good health throughout both experiments. Mider and Morton (*4*) observed that their C57BL mice looked ill after 12 biweekly paintings of a 0.5 percent solution of methylcholanthrene in benzene, and Horton and Denman (*23*) have again emphasized the fact that the health of experimental animals affects their reaction to carcinogens. The degree to which toxicity of benzene solutions of methylcholanthrene influenced the occurrence of papillomas in the C57BL mice of the present work is not apparent. Further experiments employing lower doses of carcinogen, or other vehicles, are necessary to evaluate the possibility.

Two other observations made in the course of this work deserve comment. The first was the lack of a strong correlation between the size of a skin lesion and its degree of malignancy. Most papillomas were permitted to grow until they demonstrated gross signs of malignancy, and these usually proved to be carcinomas or sarcomas when examined histologically. However, on various occasions mice were killed early because of ill health or the presence of a mammary tumor when their skin lesions were from 2 to 4 mm. in diameter, and most of these, when examined microscopically, were carcinomas. The second was the variation in response of individuals within an inbred strain. This variation is most pronounced in resistant strains and in these experiments strain C57BL showed the most variability. In the first experiment, the first papillomas arose in a strain C57BL mouse only 4 weeks, and the last papillomas 35 weeks, after the first painting. In the second experiment, the first papilloma was found 8 weeks and the last 65 weeks after the first painting. In contrast, strain I mice developed papillomas in 8 to 25 weeks in the first experiment and in 8 to 27 weeks in the second.

Finally, applications of croton oil to the area of skin painted previously with methylcholanthrene increased the incidence and decreased the average time of appearance of papillomas in treated mice as compared with untreated controls. The effect of croton oil was most pronounced in females of strains C3H and I; 11 of 12 mice treated with croton oil and none of 16 untreated controls developed papillomas. Thus, the ability of croton oil to accelerate the appearance of skin tumors was confirmed. Whether this ability of croton oil is the result of its promoting action on dormant tumor cells (*25*) or its carcinogenic property (*26,27*) is unknown.

Summary

Two experiments were performed in which inbred mice received weekly applications to unshaven skin of a 0.25 percent solution of 3-methylcholanthrene in benzene.

In the first, mice of strains A, BALB/c, C57BL, C3H, DBA/2, I, and RIII received 20 applications. This dose of carcinogen was too large to reveal significant strain differences in response, but animals of strain I developed papillomas earlier and in greater numbers than other strains. All strains showed a high incidence of malignant tumors.

In the second, mice of strains BALB/c, C57BL, C3H, I, and RIII received 10 applications. Strain I developed papillomas earlier and strain C57BL later than other strains. Strains BALB/c, C3H, and RIII were of intermediate susceptibility. All strains again revealed a high incidence of malignant tumors.

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The Influence of Differences between Reciprocal Hybrids upon Tumor Transplantation and Growth[1]

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Previous publications from this laboratory (*1–3*) reported a maternal influence, presumably the mammary tumor agent, that enhanced the transplantability and growth rate of a certain tumor and decreased the survival time of tumor-bearing mice. It appeared reasonable to presume that the presence or absence of the agent in the host accounted for the observed differences, but a small element of doubt arose from the fact that reciprocal hybrids were used as hosts for the tumor and, although reciprocal hybrids are genetically alike in general (*4*), the known differences between them prevented assurance that our observations were ascribable to the agent alone. Discussion of this point and references to related observations by others can be found in the publications cited above.

The work to be reported here was done in order to observe the influence, if any, of differences between reciprocal hybrids upon growth or transplantability of 6 tumors of mice, including the tumor used previously. The results indicate that, with these materials, the effect of such differences is slight in some cases, or imperceptible in others, and would not account for differences of the degree previously reported. However, the data presented some interesting features which, if they can be corroborated, point to influences of a sort not previously known.

Materials and Methods

The hosts were special F_2 hybrids derived from strains C3H/He, C3Hf/He (a subline free from the agent), and BALB/cAn (hereafter referred to as strains C3H, C3Hf, and C, respectively). First, F_1 hybrids of 4 classes, *i.e.*, 2 reciprocal pairs, were obtained; namely, C3H♀ × C♂, C♀ × C3H♂, C3Hf♀ × C♂, and C♀ × C3Hf♂. By interbreeding such reciprocal F_1 hybrids, 6 classes of what might be called "doubly reciprocal" F_2 hybrids were obtained (8 classes could have been obtained but only 6 were used). The classes and their special attributes are shown in table 1. In the table, "prenatal extrachromosomal influences" is used to indicate any cytoplasmic or intra-uterine influence that may have been transmitted by the maternal grandmother through the mother; those influences

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cannot be separated in these animals. The other headings are self-explanatory.

Note that in each class of animals the total genetic endowment was derived, on the average, half from strain C and half from strain C3H, and that the latter half was derived equally from the 2 sublines of the strain.

The 6 tumors comprised 3 morphologically similar, spontaneous mammary carcinomas of strain C3H origin, C3HA, C3HD, and C3HBA [the last has been described (*3*)]; a plasma-cell neoplasm, SS, originating in strain C3H (obtained from Dr. Thelma B. Dunn); a sarcoma, DBA49, originating in strain DBA/2 and previously described (*5*); and a squamous-cell carcinoma of the skin, B365MCA, originating in strain C (obtained from Dr. W. R. Bryan).

The technical details were generally similar to those described in publications cited and will not be repeated here. All inoculations were 0.025 cc. of a 50 percent suspension placed subcutaneously. The only important variations were that, owing to a shortage of animals, tumors SS and B365MCA were inoculated into males only and tumor DBA49 was inoculated into females only. Also, tumor B365MCA has such a high rate of regression in these animals that it was necessary to base our observations on the number of tumors present at 2 weeks in order to have a sufficient number of positive observations. For the other tumors, that was not necessary and they were evaluated on the basis of lethality.

Groups of animals used for comparison were balanced as to age and sex (except as noted). A total of 763 hosts were inoculated; 170 received tumor C3HA, 239 received tumor C3HD, 119 received tumor C3HBA, 55 received tumor SS, 60 received tumor DBA49, and 120 received tumor B365MCA.

Results and Discussion

The results are given in detail in tables 1 and 2. Each tumor grew in its characteristic way, as expected, but, except for differences between tumors, there were no statistically significant differences in the results that could be correlated with any known reciprocal genetic attribute of the hosts. In addition to enumerating all the tumors, we excised and weighed tumors C3HBA and C3HD, as had been done in previous work, but the weights also showed no significant differences. However, the results suggest some useful conclusions and some interesting minor influences.

Tumor C3HBA grew somewhat better in animals carrying the agent—73 percent takes *versus* 66 percent when all animals are considered. (These figures are not shown directly but can be extracted from table 1.) This difference should not be compared to those previously reported because *a*) the tumor has gone through about 120 transplant generations since the first report, *b*) the hosts are different, and *c*) one cannot be sure that the agent was entirely absent from hosts of classes II, IV, V, and VI because of the possibility of irregular transmission of the agent by males (*6,7*). (The last complication was less important in previous work because

TABLE 1.—*Results of inoculating 6 different tumors into 6 classes of hosts* *

Hosts	Classes						
	I	II	III	IV	V	VI	
Genetics							
♀	(C3H × C)	(C3Hf × C)	(C3H × C)	(C3Hf × C)	(C × C3Hf)	(C × C3Hf)	
×	×	×	×	×	×	×	
♂	(C × C3Hf)	(C × C3H)	(C3Hf × C)	(C3H × C)	(C × C3H)	(C3H × C)	
Agent	+	−	+	−	−	−	
Prenatal extrachromosomal influences	C3H	C3Hf	C3H	C3Hf	C	C	
Paternal chromosome							
y in ♂	C3Hf	C3H	C	C	C3H	C	
x in ♀	C	C	C3Hf	C3H	C	C3H	
Tumor and strain of origin							Totals by tumor
C3HA C3H	**11/30** **(37)**	**10/30** **(33)**	12/24 (50)	10/29 (34)	**7/30** **(23)**	6/27 (22)	56/170 (33)
C3HD C3H	**28/40** **(70)**	**31/40** **(78)**	26/40 (65)	24/39 (60)	**28/40** **(70)**	26/40 (65)	163/239 (68)
C3HBA C3H	**15/20** **(75)**	**15/20** **(75)**	14/20 (70)	11/20 (55)	**13/20** **(65)**	13/19 (65)	81/119 (68)
SS C3H	**3/9** **(33)**	**6/10** **(60)**	4/10 (40)	2/8 (25)	**6/10** **(60)**	3/8 (38)	24/55 (44)
DBA49 DBA	5/10 (50)	8/10 (80)	**7/10** **(70)**	**8/10** **(80)**	7/10 (70)	**6/10** **(60)**	41/60 (68)
B365MCA C	8/20 (40)	7/20 (35)	**10/20** **(50)**	**9/20** **(45)**	7/20 (35)	**13/20** **(65)**	54/120 (45)
Totals by class	70/129 (54)	77/130 (59)	73/124 (59)	64/126 (51)	68/130 (52)	67/124 (54)	

* The columns show the characteristics of each class of hosts and, below that, the results for each tumor in that class of hosts. Successful inoculations are shown both as a fraction of the total number inoculated and, in parentheses, as percent. Combinations in which the strain of the tumor and the strain of the paternal grandfather of the host correspond (DBA is considered equivalent to C; *see* text) are shown in boldface type; noncorresponding combinations are in lightface type.

TABLE 2.—*Results of inoculating 6 tumors into reciprocal hybrids* *

Tumors		Genetic attributes of hosts					
		Strain of paternal chromosome				Prenatal extra-chromosomal influences	
		y in ♂		*x* in ♀			
Designation	Strain of origin	C3H	C	C	C3H	C3H	C
C3HA	C3H	18/60 (30)	18/52 (35)	10/30 (33)	10/28 (36)	20/59 (34)	13/57 (23)
C3HD	C3H	43/60 (72)	40/59 (68)	44/60 (73)	36/60 (60)	55/79 (70)	54/80 (68)
C3HBA	C3H	23/30 (77)	21/29 (72)	20/30 (67)	17/30 (57)	26/40 (65)	26/39 (67)
SS	C3H	15/29 (52)	9/26 (35)			8/18 (44)	9/18 (50)
DBA49	DBA/2			20/30 (67)	21/30 (70)	16/20 (80)	13/20 (65)
B365MCA	C	22/60 (37)	32/60 (53)			16/40 (40)	20/40 (50)

*Results are grouped with respect to the reciprocal genetic attributes of the hosts. Successful inoculations are shown both as a fraction of the total number of hosts of each kind and, in parentheses, as percent. None of the differences are significant, except those between tumors. (DBA is considered equivalent to C· *see* text.)

of the observed effect of foster nursing.) The data can be taken only as an indication that the tumor is still dependent upon the agent in some degree. The transplantability of tumor C3HA also may be somewhat enhanced by the presence of the agent (43 percent *vs.* 28 percent; figures can be derived from table 1).

Table 2 shows the data arranged to facilitate direct comparisons for each tumor with respect to 3 reciprocal attributes of the hosts. The phrase "prenatal extrachromosomal influences" is again used to indicate all cytoplasmic and intra-uterine influences from the mother and maternal grandmother. The reader may note some interesting trends in the data, especially the tendency for 4 of the tumors to grow in more males carrying a *y* chromosome of the strain corresponding to the strain of origin of the tumor. However, none of the differences are statistically significant. The greatest difference is that shown for tumor B365MCA growing in males; in that case $P = 0.07$. For all other differences (except that between tumors) the value of P is greater than 0.10. Furthermore, as will be shown, the interpretation of a simple association, as suggested by the data, cannot be supported, even though other statistical supporting evidence can be deduced.

There is a complex, over-all aspect of the data of table 1 that is statistically significant. Among the 6 classes of hosts, the paternal grandfather was of strain C3H in 3 classes and of strain C in 3 classes; in table 1 this can be read as the source of the *y* chromosome. Among the 6 tumors, 4 originated in strain C3H, 1 in strain C, and 1 in strain DBA/2. The latter tumor belongs to the same *H-2* histocompatibility group (*8*) as strain C and in this analysis it will be treated as strain C. Thus in the table there are 18 results for combinations in which the strain of the tumor and of the paternal grandfather were the same and 18 results for combinations

in which the strains were different. If the results for each tumor are ranked (not for the classes of hosts—the ranking among them reflects expected differences in the tumors), there is a statistically significant [3] tendency for the higher percentages to occur when the strains correspond. The lowest number of successful grafts with all 6 tumors occurred in a combination in which the strains did not correspond. For 4 tumors the highest number of successful grafts occurred when the strains of origin corresponded; for 1 tumor there was a tie between corresponding and noncorresponding combinations, and for 1 tumor the highest number occurred in a noncorresponding combination. However, some of the groups were small, even though the total number of animals used was large, and the general distribution of the results shows some inconsistency. We were impressed by the fact that this effect was seen repeatedly in our material but believe that final decision should wait for confirmation in other situations.

We have no explanation for an influence correlated with the paternal grandfather—if it is really present. Inspection of the data of table 2 shows that the presence of the *y* chromosome in the male hosts is not the sole influence because the results with the 3 tumors that were inoculated into both male and female hosts indicated that, when the males with a *y* chromosome corresponding to the origin of the tumor yielded superior results, their sisters did also, and *vice versa*. (In identifying these groups note that the *x* and *y* chromosomes contributed by the father to his sons and daughters were from alternate strains.)

Consideration was also given to the fact that in the hosts the average percentage of total chromatin from one strain or the other differs slightly between groups because of the difference in size of the *x* and *y* chromosomes and the 2 sources of each. Thus, in the hosts, it could be assumed that, on the average, chromatin from 2 sources was equally distributed on 19 chromosomes but on the 20th the chromatin might be entirely of one strain or the other, or varied proportions of each. If only male hosts are considered, the data suggest that the tumor grew better in hosts having a higher percentage of chromatin (and whatever may go along with it) of the same strain as the tumor. However, if all hosts are considered, this influence is not consistent.

In sum, there are small differences in the data which, taken together, comprise a trend that we are unable to correlate with anything except the strain of the paternal grandfather. We know of no way to rationalize such an influence. Despite the outcome of statistical analysis, it is possible that there was some unsuspected, accidental influence. The uncertainty can be resolved only by confirmation or contradiction based upon other material.

Two useful deductions can be made from the data. First, the marked enhancing effect of the presence of the agent upon tumor growth, previously reported, was indeed the influence of the agent and was not ascrib-

[3] We are indebted to the Biometry Section, National Cancer Institute, for assistance in the statistical evaluation of our data. Several methods were applied and we concluded that the value of *P* was about 0.02, but it varies above and below that value according to the method used.

able to other reciprocal differences between the hosts. Theoretically, this leads to interesting speculations when one recalls that the agent was presumably the original carcinogenic stimulus for the tumor—speculations that we need not go into here. The second deduction is that, in this sort of work, the differences between reciprocal hybrids may produce small differences but generally do not produce large differences—if one can generalize from these data. In weighing the latter point it is worthy of note that the tumors represented 3 strains of origin, 4 morphologic types, and 4 degrees of transplantability (*i.e.*, factorial requirements of about 1, 3, 4, and 7), and among both the hosts and the tumors some contained the agent and some did not.

Summary

Previous work had indicated that, in reciprocal hybrid hosts, the presence or absence of the mammary tumor agent exerted a strong influence upon the growth of a certain tumor. This work was done to determine whether the subtle genetic differences between reciprocal hybrids accounted for the previous observations. Six tumors of varied types were inoculated into 6 classes of "doubly reciprocal" F_2 hybrids derived by interbreeding 4 classes of reciprocal F_1 hybrids. The results showed no significant differences that could be correlated with known reciprocal genetic differences and indicated that the previous observations were ascribable to the action of the agent. However, there was a significant, complex correlation involving the strain of the tumor and that of the paternal grandfather—a correlation for which no explanation is evident and upon which judgment is postponed. The correlation did not appear explainable by the distribution of the *y* chromosomes nor by the distribution of the average percentage of chromatin from one strain or the other.

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The Purification and Properties of Deoxyribonuclease and Ribonuclease from Normal and Neoplastic Tissues[1]

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Earlier studies (*1*) showed that the deoxyribonuclease (DNase) and the ribonuclease (RNase) which were present in our catheptic preparations from calf spleen and thymus differed from the intestinal and pancreatic nucleases in their *p*H optima, in their activation with magnesium ion, and in the sensitivity of the RNase to heat denaturation (*cf. 2–5*). In the present paper a procedure has been developed which permits marked purification of the DNase and RNase activities from rat liver, hepatoma N (Novikoff), hepatoma #3683 (Morris), calf spleen, and lymphosarcoma R2788 (Dunning). Comparative studies on the *p*H optima, magnesium-ion inhibition, and heat denaturation of the DNase and RNase activities from these tissue sources were made. These studies revealed definite differences in the RNase activities of liver and spleen and indicated that each tissue may have characteristic RNase activities.

Experimental Methods

Sources of enzyme preparations.—The following transplantable rat tumors were used: 1) Hepatoma #3683 was induced in A × C (Irish) rats by Dr. H. P. Morris by feeding 2-diacetylaminofluorene, and was of liver-cell origin. Large intraperitoneal transplants of this tumor produced solid and nodular growths which killed the rats in 3 to 4 weeks. 2) Hepatoma N (*6*) was induced in Sprague-Dawley-Holtzman rats by Dr. A. B. Novikoff by feeding *N, N*-dimethyl-*p*-phenylazoaniline. This hepatoma was also of liver-cell origin. The rats died within 6 days after a small intraperitoneal implantation of hepatoma N, which grew in small nodules and was accompanied by massive intraperitoneal hemorrhages. 3) Lymphosarcoma R2788, a spontaneous reticulum-cell-type tumor was found in an A × C (Irish) rat by Curtis and Dunning (*7*). Two small pieces of this tumor, inoculated intraperitoneally, killed the rats in about 2 weeks.

The pooled livers of normal rats and the spleens from freshly slaughtered calves supplied the normal tissues.

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Assays of DNase and RNase Activities

The substrates.—The DNA substrate was highly polymerized calf-thymus nucleate, prepared according to the method of Hammarsten (*8*), as modified by Taylor, Greenstein, and Hollaender (*9*).

Rat-liver RNA was prepared according to the method of Grinnan and Mosher (*10*). Using a concentration of 0.46 percent RNA they obtained an $S_{20,w}$ of 9.5, with correction for the viscosity of the solvent, and calculated the molecular weight to be 264,000. With the same concentration, and assuming that the RNA preparation used in these experiments had the same shape and degree of hydration, a molecular weight of 238,000 was estimated. The major peak, representing 80 percent of the total, had an $S_{20,w}$ of 8.9; there were 2 smaller peaks, one representing 12 percent, which sedimented faster, and the other comprising 8 percent, which sedimented more slowly.[3] Less than 0.6 percent of the total absorption of the RNA substrate was soluble in ice-cold 3 percent perchloric acid.

The digests.—Each 6 ml. of digest contained 2 ml. buffer, 2 ml. enzyme solution, and 2 ml. of a water solution of 1 mg. DNA or RNA. The buffers used were solutions of 0.2 *M* disodium methylarsonate in 0.2 *M* sodium acetate, which were adjusted with 10 percent acetic acid to the desired *p*H. This buffer system was suggested by the cacodylate buffers used by de Lamirande, Allard, and Cantero (*11*). Preliminary experiments showed that the disodium methylarsonate buffers permitted more rapid hydrolysis of the rat-liver RNA and the thymus DNA than the acetate-borate-cacodylate or the phosphate-borate-cacodylate buffers.

RNA digestion.—After 5 and 10 minutes at room temperature, 24° C., 2 ml. aliquots of the RNA digest were added to 2 ml. ice-cold 6 percent perchloric acid in centrifuge tubes. The tubes were then centrifuged in the cold, preferably with a horizontal head, for 20 minutes at 2000 r.p.m., and the extinctions of the supernatant solutions were read at 260 mμ in a Beckman model DU spectrophotometer.

DNA digestion.—The DNA digests were kept at 39° C. in a water bath, and 2 ml. aliquots were removed at 10 and 20 minutes and added to 2 ml. of cold 6 percent perchloric acid in centrifuge tubes. As with the RNA digests, the insoluble nucleic acid was separated by centrifugation at 2000 r.p.m. for 20 minutes, and the extinctions of the supernatant solutions were read at 260 mμ.

The final extinction values used for calculating the rate constant were obtained after digestion of both substrates for 18 hours.

In the studies on the effect of *p*H upon the nuclease activities, the digests contained 2 ml. of 0.2 *M* methylarsonate-acetate buffer of known *p*H, and the *p*H's were checked after the digestion period. The increases in absorption, at 260 mμ, of the perchloric-acid-soluble products of digestion per mg. N in the enzyme solutions were calculated and plotted directly on the *p*H activity curves.

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[3] We are indebted to Mr. Fred Gutter for these molecular-weight determinations.

Determination of Rates of DNase and RNase Activities

Over a wide range of concentrations the rates of both the DNase and RNase activities could be determined by measuring, at intervals, the extinctions at 260 mμ of the nucleic acids and their digestion products, which were made soluble in cold perchloric acid by the enzymic activity. This rate of increase of extinction, when plotted as a logarithmic function, was nearly proportional to the concentration of the enzymes under the conditions chosen for the assays. K_e, the rate constant for a first-order reaction, measures the amount of nuclease activity in terms of the fraction of the remaining substrate which is hydrolyzed during each hour of digestion. It was obtained by plotting, against time, on logarithmic paper, the difference between the final extinction reading, E_f, and the extinction reading at time t, E_t. From the slope of the line K_e, the first-order rate constant could be determined. One unit of activity is the amount of enzyme required to hydrolyze 63 percent of the RNA or DNA present in 1 hour.

In preliminary experiments we found that the RNase activity was not directly proportional to the enzyme concentration after 10 minutes' digestion and that the products of the reaction were inhibitory (*cf. 12,13*). It was necessary, therefore, to use short digestion periods and low substrate concentrations, maintaining the digest at room temperature. Under these conditions the 10-minute digestion period represented from 10 to 40 percent of the total DNase reaction and from 20 to 50 percent of the total RNase reaction.

Fractionation Procedure

The DNase and RNase activities obtained in the principal steps of a typical fractionation are shown in table 1. A model PR-2 International refrigerated centrifuge was used, and the fractions were kept at 2 to 5° C. during the preparation. From 200 to 1500 gm. of frozen tissue were suspended in 2.5 volumes of cold 0.325 *M* KCl and emulsified in a Waring blendor for 15 minutes. The emulsion was filtered through gauze (fraction I) and centrifuged at 1500 r.p.m. (600 × g) for 45 minutes. The sedimented nucleoproteins and cellular debris were washed twice with the cold, dilute KCl. The wash fluids were added to the supernatant solution and the mixture (fraction II) was dialyzed against cold (9°) running tap water for 48 to 66 hours (fraction III). The coarse protein precipitate which appeared was separated by centrifugation and discarded. Solid ammonium sulfate was added to the supernatant solution to make it 40 percent saturated (28 gm. per 100 ml.), and the precipitate was allowed to settle for 18 hours in the cold. After centrifuging at 4000 r.p.m. (2400 × g) for 30 minutes, the sediment was discarded, and the *p*H of the supernatant solution was adjusted to 3.5 with 20 percent acetic acid. This step in the procedure was found to separate most of the catheptic activity—in the precipitate—from the nucleases in the supernatant solution, and thereafter the catheptic and nuclease fractions were treated separately.

TABLE 1.—*Steps in the purification of DNase and RNase activities in a hepatoma N homogenate**

Fraction	DNase		RNase			
			*p*H 5.8–6.2		*p*H 7.8–8.2	
	Total units†	Units per mg. N	Total units	Units per mg. N	Total units	Units per mg. N
I Homogenate	83, 100	14	61, 300	10. 3	38, 300	6. 5
II After removal of nucleoproteins	81, 500	16	44, 300	8. 7	37, 000	7. 3
III Supernatant after dialysis	14, 600	9. 5	32, 600	21. 3	49, 500	32. 3
IV Nuclease precipitate dialyzed	22, 900	337	19, 500	300	19, 100	280
V First alcoholic precipitate	4, 480	514	8, 000	482	18, 700	520
VI Second alcoholic precipitate	4, 250	622	9, 100	991	20, 200	2, 189
VII Lyophilized preparation	4, 150	455	7, 300	779	17, 700	1, 900

* Three hundred sixty-nine grams of hepatoma N tissue yielded 73 mg. of lyophilized nuclease preparation.
† See text for definition of unit.

The nucleases were concentrated in the supernatant solution by adjusting the *p*H of the solution to 8.0–8.3 with 2.5 *N* NaOH and then saturating with solid ammonium sulfate. After standing overnight in the cold, a reddish-brown precipitate formed which, upon centrifugation and dialysis, yielded a fraction with greatly increased specific DNase and RNase activities (fraction IV, table 1). Further purification was effected by adjusting the *p*H of the solution to 4.2 in 0.01 *M* sodium citrate and adding 2 volumes of 95 percent ethanol at 2°. The precipitate was extracted with cold water and the alcoholic precipitation was repeated once. Further purification of the nuclease fraction by cold-ethanol precipitation resulted in some increases in specific activities, which were usually accompanied by considerable loss in total activities.

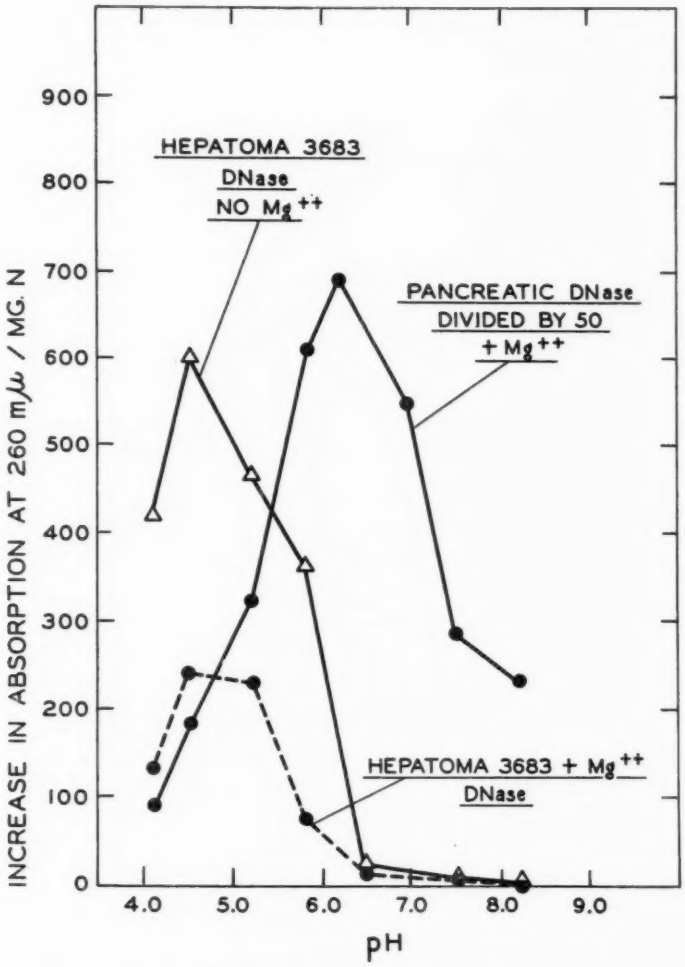
Results

DNase Activities

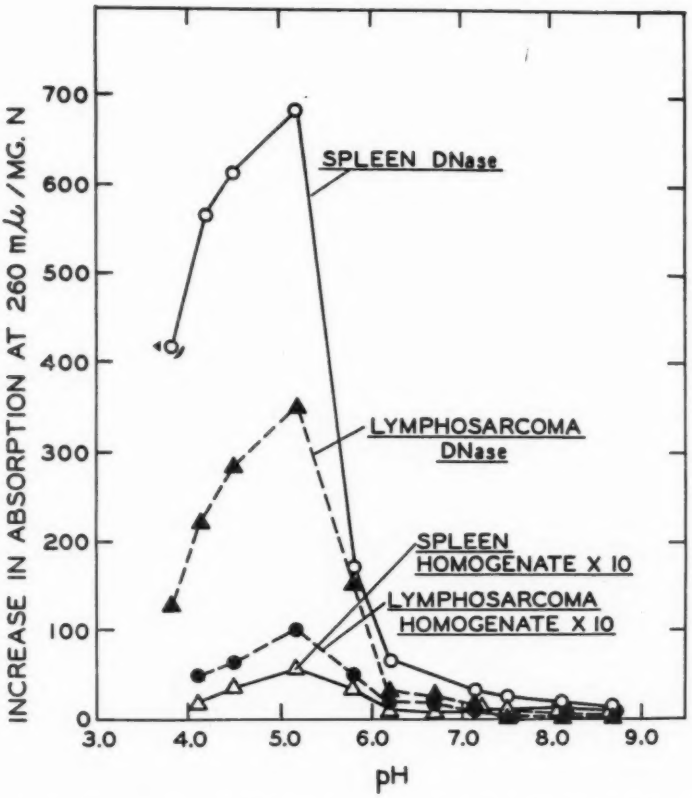
Effect of p*H.*—All the homogenates and purified fractions of liver, hepatoma, spleen, and lymphosarcoma contained a DNase activity with an optimum in the *p*H range 4.5 to 5.2. The *p*H optimum was found to shift during the digestion periods of 10 and 20 minutes, and a range of optimal activity is therefore given (text-figs. 1 and 2). There was very

slight indication of another optimum at *p*H 7.8 to 8.2 in some preparations. Using the same assay procedure that was used for hepatoma #3683, a solution of 0.3 μg. per ml. of crystalline pancreatic DNase (Worthington Biochemical Corporation), when activated with 0.005 *M* Mg^{++}, showed optimal activity at *p*H 6.2. The same concentration of magnesium ion which activated the pancreatic DNase inhibited the DNase activities of the normal and neoplastic hepatic tissues.

Rates in normal and neoplastic tissues.—The extent of purification of the DNase can be seen in table 2 by comparing the specific activities of the



TEXT-FIGURE 1.—DNase activities per mg. N are given in increases of absorption at 260 mμ of the perchloric-acid-soluble products of digestion after 10 minutes' incubation at 39° C.



TEXT-FIGURE 2.—DNase activities per mg. N are given in increases of absorption at 260 mμ of the perchloric-acid-soluble products of digestion after 10 minutes' incubation at 39° C.

purified fractions with those of the homogenates. The purification is about 100-fold for the spleen and lymphosarcoma and 200- to 300-fold for the hepatomas and normal liver.

The calf-spleen and rat-lymphosarcoma tissues showed much higher rates of DNase activity than the hepatic tissues, both in the homogenates and in the purified preparations.

RNase Activities

Effect of pH.—Two RNase activities were evident in the *p*H activity curves of the homogenates and of the purified fractions from the hepatic tissues, as seen in text-figures 3 and 4. The acid and alkaline *p*H optima of RNase activity have been reported previously by de Lamirande *et al.* (*11*) and Roth (*14*). It is interesting to note that the relative activities of the peaks in the homogenate *p*H curves were reversed in the purified

TABLE 2.—*Deoxyribonuclease activities**

Source of enzyme	Homogenate (*units/mg. N*)	Lyophilized preparation (*units/mg. N*)
Normal rat liver	*0. 82* (0. 53–1. 00)	*308* (274–331)
Hepatoma #3683	*2. 72* (1. 36–3. 88)	*592* (412–646)
Hepatoma N	*1. 65* (1. 29–2. 02)	*418* (390–445)
Calf spleen	*13. 14* (9. 36–17. 16)	*1407* (1203–1646)
Rat lymphosarcoma	*10. 29* (7. 2–18. 47)	*1046* (738–1373)

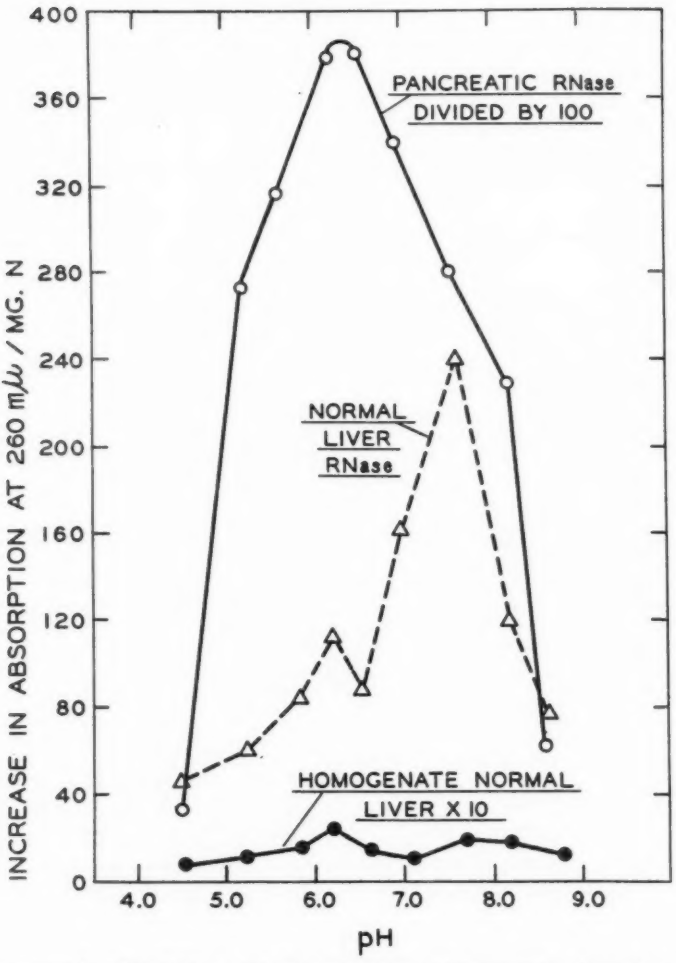
*Rates of activity are expressed as units per mg. N in the enzyme preparation. See text for definition of unit. Figures in italics are average values of 3 or more preparations; figures giving range of values are in parentheses. The digestions were in *p*H 4.5 and 5.2 buffer and were incubated at 39° C.

fractions from normal liver and hepatoma N, while the higher acid peak of the hepatoma #3683 homogenate was also present in the purified fractions from this tumor. Much of the increase in activity in the alkaline range occurred during the first dialysis of homogenates (table 1).

Kunitz (*15*) found that crystalline pancreatic RNase digested yeast nucleic acid over a wide *p*H range with an optimum at *p*H 7.8. In text-figure 3 the optimum at *p*H 6.5 is seen for the digestion of rat-liver RNA by crystalline pancreatic RNase (Worthington Biochemical Corporation). Using the same assay procedure, the RNase activities from hepatic tissue showed optima at *p*H 5.8 to 6.2 and at *p*H 7.8. It is possible that the high activity of the secretory pancreatic RNase may mask the activity of a much weaker RNase acting at a lower *p*H, since Martin and Porter (*16*) were able to distinguish 2 enzymatically active components from a crystalline RNase by chromatographic techniques. The *p*H optima of the 2 components were not reported.

The homogenates and purified fractions from normal calf spleen and rat lymphosarcoma showed a definite optimal activity at *p*H 5.5 to 5.8, corresponding to the acid optimum of the hepatic tissue RNase (text-fig. 5). In marked contrast to the normal liver and hepatoma preparations, the spleen and lymphosarcoma preparations had relatively low activity in the alkaline *p*H range.

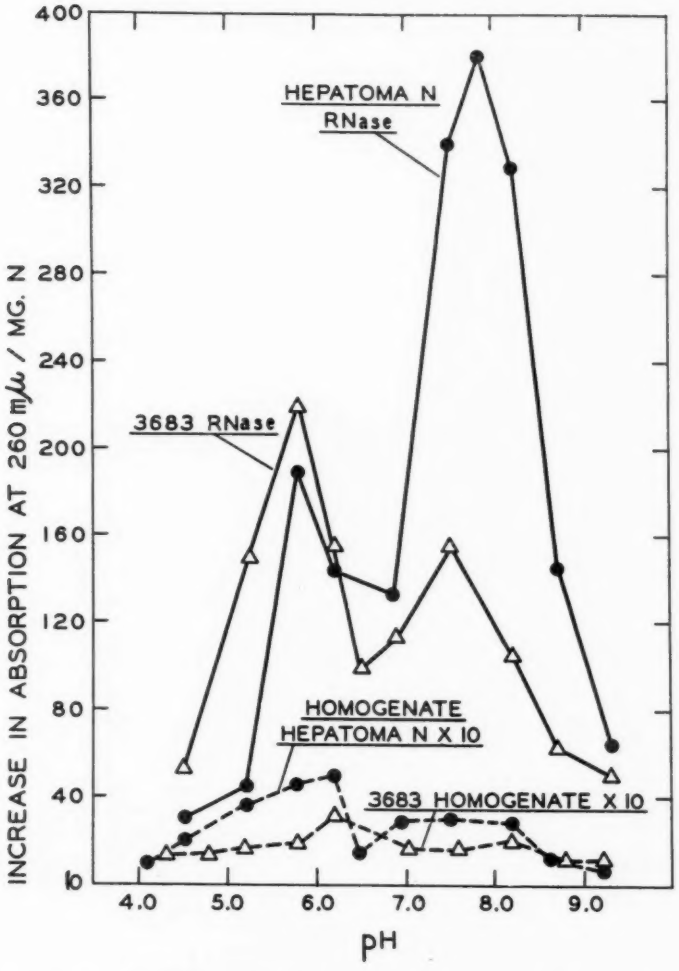
Rates in normal and neoplastic tissues.—Table 3 shows the specific activities of the homogenates and purified fractions of the normal and neoplastic tissues studied. The hepatoma homogenates showed higher specific activities than the corresponding activities of the homogenates of normal rat liver at both *p*H optima. The highest specific activities were found in the alkaline RNase of the hepatoma N preparation, whereas the alkaline RNase activity of the hepatoma #3683 preparation was not increased over that of the normal liver preparation.



TEXT-FIGURE 3.—The RNase activities are given in increases in absorption at 260 mμ of the perchloric-acid-soluble products of digestion after 5 minutes' incubation at room temperature.

The acid RNase of the spleen and lymphosarcoma was much more active than the alkaline RNase in the homogenates and in these purified fractions.

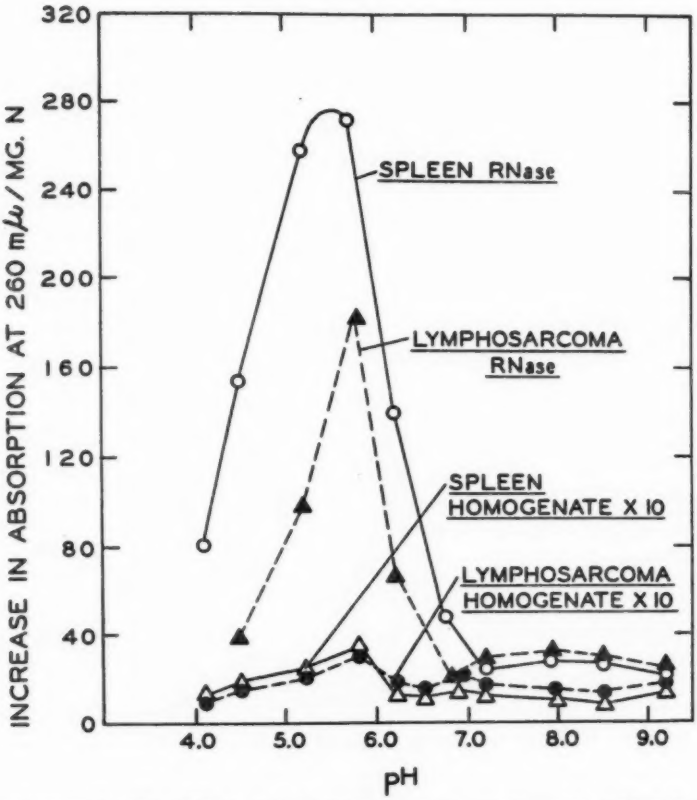
Effect of heating.—The RNase activities of the purified preparations from normal rat liver and the 2 hepatomas showed considerable resistance to heating (table 4). The activities at the acid and the alkaline *p*H optima, tested after heating, showed approximately the same percentage of the original activities. The conditions chosen for this heating experiment were those used by McDonald (*17*), who found that 77 percent of the original activity remained when crystalline pancreatic RNase (1 mg. per ml.) was heated to 100° for 15 minutes in 0.1 *N* acetic acid.



TEXT-FIGURE 4.—The RNase activities are given in increases in absorption at 260 mμ of the perchloric-acid-soluble products of digestion after 5 minutes' incubation at room temperature.

In contrast to the rat-liver and hepatoma RNase activities, the calf-spleen and rat-lymphosarcoma RNase activities were very sensitive to heat. Under the same conditions of heating at 100° for 15 minutes, the DNase activities of all the tissue preparations were destroyed.

Heavy precipitates formed when our homogenates (1 gm. per 100 ml.) were heated to 100° in 0.1 *N* acetic acid, and very irregular and inconsistent results were obtained when the RNase activities were assayed after adjustment to *p*H 5.8 and *p*H 7.8. Roth (*18*) reported that the RNase



TEXT-FIGURE 5.—The RNase activities of the homogenates and purified fractions from calf spleen and rat lymphosarcoma are given in increases in absorption after 5 minutes' digestion at room temperature.

TABLE 3.—*Ribonuclease activities**

Source of enzyme	Homogenate		Lyophilized preparation	
	*p*H 6.2 (*units/mg. N*)	*p*H 8.2 (*units/mg. N*)	*p*H 5.8–6.2 (*units/mg. N*)	*p*H 7.5–8.2 (*units/mg. N*)
Normal rat liver.....	*5.5* (5.2–5.8)	*2.9* (2.3–3.6)	*329* (317–365)	*498* (433–712)
Hepatoma #3683.....	*13.8* (10.8–19.1)	*9.6* (6.9–14.5)	*442* (350–451)	*439* (406–479)
Hepatoma N.....	*9.9* (9.0–10.3)	*6.4* (4.8–8.2)	*735* (514–914)	*1633* (1207–1900)
Calf spleen.....	*17.2* (12.3–26.2)	*3.76*	*1111* (1006–1212)	*142* (107–187)
Rat lymphosarcoma.....	*9.96* (9.5–10.20)	*3.24* (3.0–3.8)	*526* (486–556)	*82* (65–94)

*Rates of activity are expressed as units per mg. N in the enzyme preparation. See text for definition of unit. Figures in italics are average values of 3 or more preparations; figures giving range of values are in parentheses.

TABLE 4.—*Effect of heating on RNase activities of purified fractions*

Source of enzyme	Temperature (° C.)	Percentage of original RNase activity after heating *	
		*p*H 5.8	*p*H 7.5
Normal rat liver	70	71	82
	100	64	65
Hepatoma #3683	70	71	73
	100	60	61
Hepatoma N	70	89	94
	100	80	76
Calf spleen	70	34	—
	100	10	
Rat lymphosarcoma	70	57	—
	100	23	

*The average percentages of the units of original activity left after heating 3 or more preparations of the enzymes for 15 minutes in 0.1 *N* acetic acid are given. After heating, the preparations were cooled and diluted, and the *p*H's were adjusted for RNase activity.

activity of rat-liver homogenates was completely inhibited when they were heated to 80° for 15 minutes in veronal buffer at *p*H 5.5.

Discussion

Recent experiments have shown that more than one DNase or RNase activity, as indicated by their *p*H optima, may be found in a given tissue. Allfrey and Mirsky (*19*) have shown that extracts of pancreatic tissue have, in addition to the very active secretory DNase with a "neutral" *p*H optimum, a DNase activity with an optimum at *p*H 5.2, which is characteristic of the endocellular DNases. Probably the "neutral" *p*H optimum corresponds to the optimum of *p*H 6.2 given in these experiments with the crystalline pancreatic DNase. Only the endocellular DNase activity with optimal activity at *p*H 4.5 to 5.2 was detected in the homogenates and the fractions from normal liver and spleen and from the hepatomas and lymphosarcoma studied here. The DNase activity of the hepatic tissues was inhibited by magnesium ions, in marked contrast to the activation shown by the secretory DNase of the pancreas (text-fig. 1). Evidence for qualitative differences in the activities of pancreatic and thymus DNase were shown by Privat de Garilhe and Laskowski (*20*) in chromatographic studies of digestion products.

The study of RNase activity is complicated by the fact that different tissues have activities with the same or different *p*H optima, which may or may not be activated by magnesium ions or inhibited by heat. In this report the liver and hepatoma preparations had RNase activities with optima at both an acid and an alkaline *p*H, while the spleen and lymphosarcoma preparations exhibited a marked acid *p*H optimum and relatively little activity in the alkaline range. Roth (*18*) has found that kidney homogenates have the 2 RNase optima characteristic of liver tissue.

Hilmoe and Heppel (*5*), with their fractionation procedure, distinguished 3 RNase activities in spleen by their *p*H optima, by activation with magnesium ions, and by their ability to hydrolyze the limit polynucleotides, or "core," produced by pancreatic digestion of yeast nucleic acid. Using an entirely different fractionation procedure, Kaplan [4] recently separated an RNase from spleen and liver which could be identified as pancreatic RNase with the use of simple substrates but which digested RNA at a different *p*H optimum.

With the cyclic nucleotides or the benzyl esters of nucleotide phosphates as substrates, Volkin and Cohn (*21*), Heppel, Markham, and Hilmoe (*22*), and Brown, Heppel, and Hilmoe (*23*) have demonstrated specific qualitative differences in the RNase activities of some spleen preparations and secretory pancreatic RNase. Heppel and Hilmoe [4] have found that our spleen-nuclease preparation was a useful tool in studies of nucleic acid structure, first, because of its very low phosphatase activity and, second, because of its specific ability to hydrolyze purine and pyrimidine 2′:3′ nucleotides (cyclic) to form only the 2′ nucleotides, while pancreatic RNase hydrolyzes only the pyrimidine 2′:3′ nucleotides to form 3′ nucleotides. Further characterization of the RNase activities of normal and neoplastic tissues with the cyclic nucleotides and the riboside 2′ and 3′ benzyl phosphate esters is under way in this laboratory. Resolution of the present complex array of RNase activities in mammalian tissues will probably be achieved by characterization of the individual enzyme activities after their separation by chromatographic techniques, as described by Sober and Peterson (*24*).

The higher specific DNase and RNase activities of the hepatoma fractions are in agreement with the results of others who have compared these activities in the homogenates and the separated nuclei (*25*) and mitochondria from hepatomas and normal liver (*26*). It is significant that these hepatomas, both of liver-cell origin, have retained the RNase activities with the 2 *p*H optima which are characteristic of the normal liver cell. Although not as closely related as these hepatic tissues, the 2 lymphoid tissues, spleen and lymphosarcoma, showed the same acid *p*H optimum for their RNase activities.

Summary

A procedure is given for the purification and concentration of the DNase and RNase activities of normal liver, spleen, 2 transplantable rat hepatomas, and a transplantable rat lymphosarcoma. The specific activities of the homogenates and the purified fractions from these tissues are given.

The DNase and RNase activities of the homogenates and preparations from these tissues were characterized by their *p*H optima, which were found to differ from the corresponding *p*H optima of crystalline pancreatic DNase and RNase. All the tissues showed a DNase activity with 1 acid *p*H optimum. The RNase activities of the tissues of hepatic origin,

[4] Personal communication.

represented by normal liver and the 2 transplantable hepatomas, were characterized by 2 *p*H optima—1 in the acid and 1 in the alkaline *p*H range. In contrast, the spleen and lymphosarcoma tissues had only 1 major RNase activity, which was in the acid range.

The RNase activities of the purified fractions of the hepatic tissues showed considerable resistance to heating, while the activities from spleen and lymphosarcoma were rapidly destroyed by heating under the same conditions.

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Tumor-Localizing Antibodies Purified from Antisera against Murphy Rat Lymphosarcoma [1, 2]

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To carry therapeutic or diagnostic amounts of radioactivity specifically to tumor sites, tumor-localizing antibodies would have to be separated from antibodies which cross-localize in normal tissues and from those inert components which make up the bulk of antibody-containing serum globulins.

In the studies so far reported by this group (*1,2*) it has been found that antisera to the Wagner osteogenic mouse sarcoma and to the solid form of the Murphy rat lymphosarcoma contain antibodies which localize not only in tumor but also in normal tissues. It was possible to concentrate localizing anti-tumor antibodies by absorbing them on sediments of the solid tumors, washing the sediment thoroughly, and eluting the absorbed antibodies at 60° C. However, there was also a concentration of antibodies which cross-localized extensively in normal tissues.

We have now been able to isolate fractions of radioiodinated anti-tumor antibodies, prepared against the Murphy rat lymphosarcoma, which localize in tumor to an amount, over and above background localization,[4] as high as 18 percent of the injected radioactive dose. Many of these fractions do not localize in normal tissues in amounts detectable by our present methods.[5] We are reporting here the methods for preparing these fractions and their localizing properties when injected into rats bearing the lymphosarcoma.

Methods of Purification

We approached the problem of obtaining highly specific tumor-localizing fractions in 2 ways: (A) fractional separation of tumor-localizing antibodies from those which cross-localize in normal tissues, and (B) separation

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[2] This study was supported in part by Atomic Energy Commission Contract AT(30-1)-1771 (Roswell Park Memorial Institute) and Contract AT(30-1)-910 (Sloan-Kettering Institute).

[3] We should like to thank Mrs. Kay Collins Pawlowski, Mr. Eugene Kanski, and Miss Mary McMahon for their technical assistance; and Dr. Alonza Johnson and Dr. Abraham Lilienfeld for their aid in statistical evaluation of the results.

[4] Background localization in the lymphosarcoma is a function of the size of the tumor and, in terms of the injected dose, ranges from 1 percent for a 2 gm. tumor in a 200 gm. rat to 9 percent for a 10 gm. tumor in a 100 gm. rat.

[5] Localizing backgrounds, in terms of the dose injected into 100 to 200 gm. rats, are: for liver, 1.2 percent; kidneys, 0.2 percent; spleen, 0.1 percent.

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of the antigens which give rise to tumor-localizing antibodies from those antigens which give rise to the cross-localizing types.

Fractional separation of antibodies.—Two processes were used initially in fractionally separating specific tumor-localizing antibodies. In the first, called *subsequent absorption*, antibodies concentrated by the previously mentioned absorption and elution method were further purified by absorbing them with sediments of normal tissues to remove the antibodies which cross-localize in normal tissues. In the second, called *prior absorption*, removal of normal-tissue-localizing antibodies by normal-tissue sediments was accomplished prior to concentration of the remaining tumor-localizing antibodies by absorption on and elution from tumor sediment.[6]

Ascites tumor used as antigen.—Since the solid form of the lymphosarcoma contained components, particularly stroma, in common with normal tissues, an ascites form of the tumor was developed to obtain tissue free from stroma and rich in tumor cells. Sediments prepared from the free ascites cells absorbed tumor-localizing antibodies selectively from antiserum globulins contaminated with cross-localizing antibodies. In addition, antisera prepared against whole ascites cells contained a very high proportion of specific tumor-localizing antibodies. These results were encouraging, since they indicated that in this particular case the production of cross-localizing antibodies was not primarily directed against tumor *per se*.

Preparations

The Murphy rat lymphosarcoma: solid, ascites, and solid-ascites forms.—The original solid line of the tumor, used in our previous investigations at Sloan-Kettering Institute in New York, was brought[7] to Buffalo in August, 1954. Since that date, the solid line has been carried in Wistar rats[8] by routine biweekly trocar implants in the right axillary region of the hosts. The tumor has grown progressively in 100 percent of these rats, causing death in about 21 days.

An ascites line of the tumor was started in April 1955, following the general method of Klein (*4*), and was transplanted weekly for 45 transplant generations. By the 8th transfer, the ascites form of the tumor was produced successfully in all hosts, and by the 11th, it was producing an average of 10 ml. of ascitic fluid per rat, with a cell count of 2×10^8 cells per ml.

The solid-ascites line of the tumor was started with ascites cells from the 21st ascites-tumor generation. One-half ml. portions of a 1:80 dilution of these cells were injected subcutaneously in the right axillary region of twenty 100 gm. rats. Solid tumors grew progressively in all 20 and reached a size of 15 to 20 gm. in about 10 days. By the 14th day there were only 2 survivors, and tumor from 1 of these was used for

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[6] Preliminary work utilizing prior-absorption procedures has been reported by Korngold and Pressman (*3*). Sediments are described on p. 519.

[7] Kindly maintained in the interim at Sloan-Kettering by Dr. Maurice Rapport.

[8] Obtained from Carworth Farms, New City, N. Y.

trocar transplantation. This line has since been maintained in the same way as the original solid tumor.

A second line of the ascites tumor was started from a tumor-cell suspension prepared from the 11th solid-ascites tumor generation. Twelve rats were injected intraperitoneally with the suspension, and all developed profuse ascites tumors with respect to volume (15 ml.) and to cell count (2.0×10^6 cells per ml.) within 7 days. Since this line proved more productive, the first was discontinued. The second line has been carried in the same way as the first, causing death in 100 percent of the hosts within 9 days.

Tissues and tissue sediments.—Normal tissues and solid tumors were obtained from perfused Wistar rats. Free ascites-tumor cells were centrifuged from the ascitic fluid and washed once with 0.9 percent NaCl. Sediments were prepared from these tissues by serial centrifugation and rehomogenization with fresh 0.9 percent NaCl (buffered to *p*H 8 with borate) to a 20 percent suspension, until supernatant fluids were clear. As many as 10 washes were necessary to prepare lung and spleen sediments; as few as 3, to prepare ascites-tumor sediment. The final products were lyophilized, powdered, and stored in screw-cap vials at 5° C.

Globulins from antisera.—Antisera were prepared in rabbits by intraperitoneal injection of homogenized tissues or tissue sediments according to the protocol previously described (*2*) for the preparation of anti-lymphosarcoma antisera. The globulin fractions were prepared according to a modification of the Kekwick (*5*) and Humphrey and McFarlane (*6*) procedures. Each globulin was obtained by adding slowly to 1 volume of serum at room temperature 2 volumes of stock sodium sulfate solution (27 gm. Na_2SO_4 per 100 ml. solution, brought to *p*H 8 with borate or phosphate). The crude globulin precipitate was centrifuged down, washed once with a sodium sulfate solution containing 2 volumes of stock and 1 volume of phosphate or borate buffer (*p*H 8, $\mu = 0.1$), redissolved in 0.5 volume buffer, reprecipitated with 0.5 volume stock sodium sulfate, and taken up in a minimum of 0.9 percent NaCl (brought to *p*H 8 with borate). The final product was dialyzed twice against 12 liter portions of the buffered saline, cleared by centrifugation, lyophilized, and stored in screw-cap vials at 5° C. The protein content of the salt-containing powder was determined by comparing the absorption, at 280 mμ, of a dissolved portion with that of a standard globulin solution. Each globulin was designated by a number and a letter to describe its source (*e.g.*, globulin lot No. 32B was obtained from the second bleeding of rabbit No. 32). Pooled lots were obtained from sera of several rabbits and normal lots from uninjected rabbits.

Radioiodination.—The general method used in previous investigations (*7*) was modified slightly—in the order of addition of reagents, amount of nonradioactive carrier iodide used, and the manner of removing free iodide from the final iodinated protein solution.

Reagents were: 1.0 *N* HCl, 1.0 *N* NaOH, 0.002 *M* KI, 0.02 *M* $NaNO_2$, and *p*H 8 borate buffer previously described (*2*). Before iodination the

following solutions were prepared in 3 separate containers from chilled stock solutions: (*A*) 1.0 ml. HCl, 0.25 ml. KI (for every 20 mg. protein), 1 ml. carrier-free I^{131} (Oak Ridge product diluted with water, usually to 4 mc. per ml.); (*B*) 1.0 ml. NaOH, 5.0 ml. borate buffer; (*C*) globulin (usually 20 mg.) dissolved in 2.0 ml. borate buffer.

In rapid succession, 0.2 ml. $NaNO_2$ was mixed in the acidified iodide solution (*A*), followed by the alkali-buffer solution (*B*). The resultant solution was then added with thorough and rapid mixing to the buffered protein solution (*C*). Unbound iodide and insoluble protein were removed by passing the solution through a 5 ml. column (prepared in a 10 ml. syringe) of Amberlite IR-4B(OH)[9] followed by 9 to 10 ml. of distilled water. [Wish, Furth, and Storey (*8*) and Talmadge *et al.* (*9*) had previously used ion-exchange procedures with success.] No dialyzable radioactivity was detected by dialysis against equal volumes of water or of 0.00001 *M* and 0.01 *M* KI. The iodination yield was 25 to 30 percent of the initial iodide and the protein recovery was approximately 95 percent; hence, an average of 1 atom of iodine was routinely bound to each protein molecule.

Absorption and elution.—These procedures were carried out as described previously (*1,2,10*), except that 40 ml. polyethylene centrifuge tubes were used in place of glass.[10] Volumes of 10 ml. (9 to 10 mg. protein) of each solution obtained from the ion-exchange column were added to weighed amounts of tissue sediments and shaken continuously for 1 hour at 37° C. The sediments were washed with borate buffer to remove entrained but unabsorbed globulins and then suspended in 10 ml. volumes of preheated borate (59° C.) and shaken continuously for 15 minutes at 59 ± 1° C. to elute a portion of the absorbed globulins. Supernatant fluids resulting from centrifugations of sediments were treated in the same way as the initial globulin solutions. Final fractions, used for *in vivo* assays, were transferred to glass serum bottles containing 0.2 ml. normal rabbit serum as the protein carrier.

The amount of radioactivity present at each stage of the absorption-elution procedure was determined as described (*see* p. 521) and from this determination the fraction of globulin was estimated. The values which appear in tables 2 to 5, under the *in vitro* headings, were obtained in this way.

Assay for localization.—Two ml. portions of each solution were injected in the tail veins of 3 Wistar rats bearing the solid-ascites lymphosarcoma. A fourth 2 ml. portion, diluted to 100 ml. with 2 percent rabbit serum in 0.9 percent NaCl, was used to determine the radioactivity of the injected dose. Rats of 100 to 180 gm., bearing tumors of 1 to 10 gm. (inoculated by trocar 6 to 7 days prior to assay), were used throughout.[11]

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[9] Rohm and Haas reagent obtained from Fisher Scientific Co.

[10] Previously, nonradioactive carrier protein had been used to reduce glass-surface absorption of the radioactive protein. Use of polyethylene here circumvented that need.

[11] For the determination of background accumulations of normal globulins as a function of tumor and rat size, rats of 90 to 280 gm., bearing tumors of 0.2 to 12 gm., were used.

Sixteen to 20 hours after injection the rats were weighed and perfused as described previously (*1,2,10*). Tissue samples weighing less than 1.0 gm. were used for the determination of radioactivity. Kidneys and spleens were counted whole, as a rule, whereas livers and large tumors were counted piecemeal. Since the distribution in liver of injected globulins was fairly uniform, the total localization could be determined by calculation from liver weight and from the radioactivity contained in 2 weighed portions of liver. Since the distribution in tumor was not uniform, the whole tumor was assayed. Also counted were two 1.0 ml. portions of the 100 ml. dilution described in the previous paragraph for each assay group, before and after the tissues from that group had been assayed.

Each experiment was carried out in 2 days—iodination, fractional separation of globulins, and injection of rats on the first and sacrifice of rats, counting of tissues, and evaluation of results on the second. Up to 36 rats could be assayed at one time, representing a total of 12 globulin fractions.

Liver, kidney, and spleen were routinely assayed along with tumor to determine the extent of cross-localization in normal tissues as well as tumor localization. Blood from each animal was also assayed, but blood values are not included in the tables which follow since they do not reflect localization properties of the injected globulins.[12]

Determination of radioactivity.—A well-type scintillation counter [13] with a sodium iodide crystal and 3-inch lead shield was used. The counter had an efficiency of 45 percent and a normal background of 140 c.p.m.[14] A 60-minute background count was obtained before and after all tissues and solutions for a given day were counted. Samples of tissues and of dilutions of pertinent solutions were counted in the well for not less than 1 minute but always long enough to accumulate 2,000 counts. Sediments, obtained at various stages of the absorption-elution process, were counted at a geometrically fixed distance from the counter head as were undiluted portions of the original iodinated globulins.

Results

Corrections for background in tissues.—When a radiolabeled preparation containing tumor-localizing antibodies is injected into a tumor-bearing host, the total radioactivity localized in the tumor is due not only to antibody but also to an accumulation of other radiolabeled proteins which produce a localizing background. Since this background is often comparable to the net localization of radioactivity due to anti-tumor antibodies themselves, it is necessary to evaluate the factors affecting the background before a determination of true localization is possible.

[12] The mean value in blood for the *in vitro* eluted globulins was 2.0 percent of the injected dose per ml. (normalized to 100 gm. rats) with a range of ± 0.6 percent. This accounts for approximately 20 percent of the dose. The mean concentrations in blood for all original globulins and supernatants containing 75 percent or more of the original globulins were 4.0 ± 1.0 percent per ml., accounting for approximately 40 percent of the total dose.

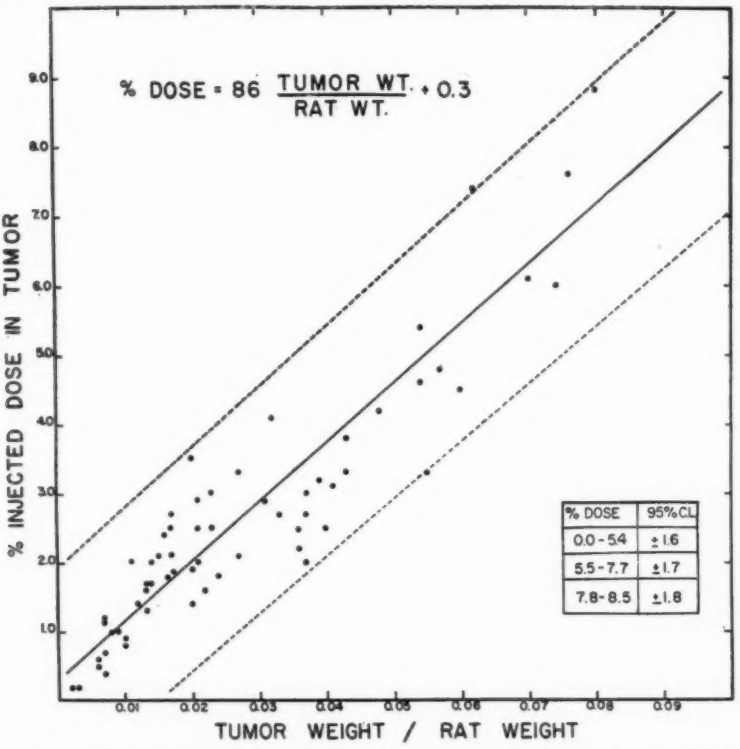
[13] Nancy Wood Co., Chicago, Ill.

[14] The least active tissue samples never counted less than eight times this normal background.

On the basis that the background in a tumor is a reflection of the concentration of radioactivity in the whole animal, we considered the total background in the tumor (percentage of injected dose) to be directly proportional to tumor weight and inversely proportional to body weight. To evaluate the effect of these variables (tumor and body weights) more precisely, we obtained data from the assays of radioiodinated globulin fractions of 4 normal and 2 anti-ovalbumin sera in 63 tumor-bearing rats. These data (percentage of injected dose accumulated in tumors *vs.* percentage of weight of tumor in the tumor-bearing hosts) were reduced by the method of least squares to the following equation:

$$\text{Percentage of injected dose in tumor} = 86\,(\text{tumor weight/rat weight}) + 0.3.$$

In addition, the hyperbolic 95 percent confidence limits for the background were determined. The plot of these data, the derived equation, and the hyperbolic 95 percent confidence limits about the percentage of dose are shown in text-figure 1.



TEXT-FIGURE 1.—Accumulation of radioiodinated nonlocalizing rabbit-serum globulin in the solid-ascites Murphy lymphosarcoma of Wistar rats as a function of tumor and body size. The *solid* line is the plot of the least-squares equation; the *broken* lines are the hyperbolic 95 percent confidence limits about the percentage of dose; the table shows these limits for a range of values.

The background accumulations in whole liver, spleen, and kidney, following similar considerations, would be constants (independent of organ and body sizes) insofar as organ weight is proportional to body weight. Moreover, these accumulations would be much less important with respect to localizing antibody than in the case of tumor. The evaluation of the backgrounds in liver, kidney, and spleen of the 63 tumor-bearing rats considered above confirmed this premise. The total mean background accumulations and 95 percent confidence limits (percentage of dose) in these rats were: liver, 1.2 ± 0.6; kidney, 0.19 ± 0.10; and spleen, 0.13 ± 0.07.

Further experiments using fractions of the globulins isolated from normal and anti-ovalbumin sera by the absorption and elution process described below also gave background localizations in every case within the limits described previously.

Values for the *net localization* in a whole tumor or in normal tissue are the differences between the observed total localization and the background accumulation. The values in the tables which follow were calculated on the basis of the above background data, as shown in table 1. Any net value falling below the upper 95 percent confidence limits of the mean background for a particular tissue was considered to represent absence in that tissue of significant amounts of true localizing antibody.

Fractional separation of tumor-localizing antibodies by absorption processes.—We have shown (*1,2*) that anti-tumor antibodies absorbed and eluted from tumor show a greater concentration of cross-localizing than of tumor-localizing components. In a pilot experiment, we indicated (*3*) that the components localizing in normal tissues could be partially removed by prior absorption with normal-tissue sediments and that the remaining tumor-localizing antibodies could then be concentrated more successfully by subsequent absorption and elution from tumor. These observations have now been extended, as shown in table 2, to whole and eluted globulins from an anti-solid-lymphosarcoma antiserum and 2 anti-solid-ascites-lymphosarcoma antisera. The prior-absorption procedure (described in more detail later) resulted in products which contained no detectable cross-localizing antibodies, even in the case of the anti-kidney antiserum used as a control. Tumor-localizing antibodies, on the other hand, were enriched.

As shown in table 2, the amount of radioactive globulin eluted from the tumor sediment represented roughly 1 percent of the total whole globulin used, whether or not the globulin contained anti-tumor antibody. In terms of the amount absorbed by the tumor sediment, about half was elutable under the conditions of these experiments if the globulin had not been previously absorbed with normal tissue to remove cross-localizing antibodies, and about two thirds if the globulin had been treated by prior absorption. Since the eluted material was contained in 10 ml. buffered saline and since each rat received 2 ml., the amount of radioactive globulin assayed in each rat represented 0.2 percent of the initial whole radioiodinated protein, or about 20γ of the initial 10 mg. A net

TABLE 1.—*Typical calculation for net localization of injected radioiodinated globulins in lymphosarcoma-bearing rats**

Group	Rat weight (*gm.*)	Tumor weight (*gm.*)	Percent injected dose localized†											
			Tumor‡			Liver			Kidney			Spleen		
			Total	Bkgd.	Net	Total	Bkgd.	Net	Total	Bkgd.	Net	Total	Bkgd.	Net
A	130	9.1	6.1	6.3	−0.2	0.9	1.2	−0.3	0.2	0.2	0.0	0.1	0.1	0.0
	180	9.7	4.6	4.9	−0.3	0.6	1.2	−0.6	0.1	0.2	0.1	0.1	0.1	0.0
	135	7.4	5.4	5.0	0.4	0.7	1.2	−0.5	0.2	0.2	0.0	0.1	0.1	0.0
	Average				0.0			−0.5			0.0			0.0
B§	119	8.1	16.5	6.1	10.4	1.5	1.2	0.3	0.2	0.2	0.0	0.2	0.1	0.1
	157	9.0	16.8	5.2	11.6	1.5	1.2	0.3	0.1	0.2	−0.1	0.2	0.1	0.1
	130	10.0	19.1	6.9	12.2	1.4	1.2	0.2	0.1	0.2	−0.1	0.2	0.1	0.1
	Average				11.4			0.3			−0.1			0.1

*Rats in Group A received whole globulin from an anti-ascites-lymphosarcoma antiserum (No. 513A). Rats in Group B received a purified antibody fraction of this globulin, which was obtained, after prior absorption with rat-lung sediment, by absorption on and elution from ascites-tumor sediment. This purification process is described in more detail in the text.

†Background localization in each tumor was calculated from the equation: bkgd. = 86 (tumor wt./rat wt.) + 0.3, as described in the text. Background localization in normal tissues was obtained as described in the text.

‡The assay tumor was the solid-ascites form of the Murphy rat lymphosarcoma.

§The fraction used contained 1.6 percent of the radioactivity of the whole globulin.

TABLE 2.—In vivo *localization in tumor-bearing rats of fractions of globulins from several rabbit antisera*

Rabbit serum	Globulin fraction	Purification of tumor-localizing antibody (% *initial radioactivity*)			Net localization of antibody* (% *injected radioactive dose*)			
		Remaining in supernatant after lung-sediment absorption	Absorbed on tumor sediment	Eluted from tumor sediment	Tumor†	Liver	Kidney	Spleen
Normal serum (pooled)	A‡	—	—	—	0.3	0.0	0.0	0.0
	B§	—	1.5	0.8	0.4	−0.5	0.0	0.0
Anti-bovine γ-globulin (pooled)	A	—	—	—	0.2	0.0	0.0	0.0
	B	—	1.6	0.8	−0.1	0.4	0.0	0.0
	C‖	91	0.9	0.6	0.5	−0.7	−0.1	0.0
Anti-whole-rat-kidney (220B)	A	—	—	—	1.2	1.1	1.3	0.3
	B	—	1.9	0.9	−0.7	10.1	5.2	1.9
	C	90	0.9	0.6	1.0	−0.2	−0.1	0.0
Anti-solid-tumor-sediment (32B)	A	—	—	—	1.6	0.2	0.0	0.0
	B	—	2.3	1.2	2.0	3.1	0.2	0.2
	C	90	1.4	0.8	2.3	0.2	0.0	0.2
Anti-solid-ascites-tumor-sediment (386A)	A	—	—	—	2.0	0.4	0.1	0.2
	B	—	2.4	1.3	3.7	4.7	0.3	0.7
	C	90	1.3	0.9	3.7	0.0	0.0	0.0
Anti-solid-ascites-tumor-sediment (391A)	A	—	—	—	1.9	0.4	0.1	0.1
	B	—	2.4	1.4	3.5	6.4	0.5	1.0
	C	91	1.3	1.0	3.3	0.1	0.0	0.0

*Net localization is percent injected dose found in whole tissues minus percent dose due to background radioactivity calculated as shown in table 1. The net localization ±95 percent confidence limits of normal globulins in 63 rats is: tumor, 0.0±1.6; liver, 0.0±0.6; kidney, 0.0±0.1; spleen, 0.0±0.1. Localization values are averaged from the assay of 3 rats.

†The assay tumor was the solid-ascites Murphy lymphosarcoma in Wistar rats.

‡Whole globulin without further treatment. Radioiodinated whole globulins (10 mg.) were diluted 1 : 100 with 2 percent rabbit serum in saline for injections in rats.

§After absorption on and elution from solid-ascites-lymphosarcoma sediment. Radioiodinated whole globulins (10 mg.) were absorbed with 10 mg. of solid-ascites-lymphosarcoma sediment. Absorbed globulins were eluted at 60° C. in 10 ml. of buffered saline (pH 8). To each eluate was added 0.2 ml. of normal rabbit serum before injection in rats.

‖After prior absorption with rat-lung sediment followed by absorption on and elution from ascites-lymphoma sediment. Radioiodinated whole globulins (10 mg.) were absorbed with 100 mg. of rat-lung sediment. Supernatants were treated according to the method for whole globulins (*see* preceding footnote).

localization of 5 percent of this material in a given tissue of a rat represented, therefore, 1γ of globulin antibody, and a net value of 0.1 percent represented 0.02γ.

Prior absorption is not the only means of obtaining removal of cross-localizing antibodies. A reverse procedure, subsequent absorption, was also used, whereby the globulin (10 mg.) was first concentrated by absorption and elution from tumor sediment and then purified by absorption with normal-tissue sediment. The same amount of normal tissue (100 mg.) was used in each procedure for removing cross-localizing antibodies, and the same amount of tumor sediment (10 mg.) for the absorption-elution step. In each process nonradioactive normal rabbit globulin (10 mg.) was added immediately after elution. Equivalent results, with respect to both yield of radioactivity and localization in rats, were obtained (table 3).

In the work so far reported, the purification of tumor-localizing antibodies has involved removal of the cross-localizing components by rat-lung sediment. In the experiment which resulted in the values shown in the first part of table 4, the purification by prior absorption was carried out with other normal-tissue sediments as well. In each case, the antibodies remaining in the supernatant fluid were concentrated by absorption on and elution from ascites-tumor sediment. It is evident that although each normal-tissue sediment removed most of the cross-localizing antibody components, some, such as lung and lymph node, also removed tumor-localizing components as well. The *in vitro* absorption with kidney sediment removed the least amount of specific tumor-localizing antibody.

Effect of tissue-stroma components in solid tumor on purification of antibody.—Since the solid forms of the lymphosarcoma contain normal-tissue components in the form of stroma which are absent in the ascites form, sediments of these solid forms in the absorption and elution process do not selectively concentrate anti-tumor antibodies alone but concentrate cross-localizing antibodies as well. This is shown in the following experiment.

The ascites-tumor sediment, when used for prior absorption (*see* table 4), removed all detectable tumor-localizing antibody and left an amount of cross-localizing antibody in the supernatant fluid which was not concentrated to any great extent by absorption and elution from the second tumor absorption (compare with localization values for the whole globulin, table 4). In contrast, 100 mg. of the solid-ascites tumor sediment removed all detectable cross-localizing antibodies from 10 mg. of globulin but left a significant amount of tumor-localizing antibody in the supernatant fluid.

This difference between ascites and solid-ascites tumor sediments is shown further in the lower part of table 4. Here spleen and liver sediments were used for prior absorption, and the unabsorbed portions were eluted from solid-ascites tumor sediment as well as the sediment prepared from free ascites cells. The results show that although a single prior absorption with normal tissues, under the conditions of these experiments, does

TABLE 3.—In vivo *localization in tumor-bearing rats of an anti-tumor preparation and 2 control antibody preparations purified both by prior- and subsequent-absorption processes utilizing lung and ascites-tumor sediments*

Antiserum	Absorption process	Purification of tumor-localizing antibody				Net localization of antibody*			
		First absorption		Second absorption					
		Sediment	Fraction taken (*percent*)	Sediment	Fraction taken (*percent*)	Tumor†	Liver	Kidney	Spleen
Anti-bovine-γ-globulin (pooled)	prior‡	100 mg. lung	supernatant (91)	10 mg. ascites	eluate (0. 6)	0. 5	−0. 7	−0. 1	0. 0
	subsequent§	10 mg. ascites	eluate (0. 7)	100 mg. lung	supernatant (0. 6)	−0. 4	−0. 5	0. 0	0. 0
Anti-whole-rat-kidney (220B)	prior	100 mg. lung	supernatant (90)	10 mg. ascites	eluate (0. 6)	1. 0	−0. 2	0. 1	0. 0
	subsequent	10 mg. ascites	eluate (0. 7)	100 mg. lung	supernatant (0. 6,	0. 0	−0. 2	0. 0	0. 0
Anti-solid-ascites-tumor sediment (389A)	prior	100 mg. lung	supernatant (90)	10 mg. ascites	eluate (1. 3)	4. 2	0. 3	0. 0	0. 1
	subsequent	10 mg. ascites	eluate (1. 5)	100 mg. lung	supernatant (1. 2)	3. 6	0. 5	0. 0	0. 1

* Net localization is the percentage of injected dose found in whole tissues minus the percentage of dose due to background radioactivity, calculated as shown in table 1. The net localization ± 95 percent confidence limits of normal globulins in 63 rats is: tumor, 0.0 ± 1.6; liver, 0.0 ± 0.6; kidney, 0.0 ± 0.1; spleen, 0.0 ± 0.1. Localization values are averaged from the assay of 3 rats.

† The assay tumor was the solid-ascites Murphy lymphosarcoma in Wistar rats.

‡ For prior absorption the globulin (10 mg.) was absorbed with a tenfold amount of rat-lung sediment; the supernatant fluid was absorbed with an equal amount of ascites-tumor sediment; the eluate from the tumor sediment was assayed for localization.

§ For subsequent absorption the globulin (10 mg.) was absorbed with an equal amount of ascites-tumor sediment; the eluate from the tumor sediment, after addition of nonradioactive normal globulin, was absorbed with a tenfold amount of rat-lung sediment; the supernatant fluid was assayed for localization.

TABLE 4.—In vivo *localization in tumor-bearing rats of anti-tumor antibodies purified by prior absorption with various tissue sediments followed by absorption on and elution from tumor sediments*

Purification*					Net localization of antibody†			
Normal-tissue prior absorption		Tumor absorption‡						
Tissue sediment	Radioactivity remaining in supernatant (*percent*)	Tumor sediment	Radioactivity		Tumor‡	Liver	Kidney	Spleen
			Absorbed (*percent*)	Eluted (*percent*)				
Kidney	94	ascites	1. 3	0. 8	8. 2	0. 0	0. 0	0. 1
Spleen	87		1. 2	0. 6	7. 0	0. 3	0. 0	0. 1
Liver	93		1. 2	0. 7	5. 0	0. 4	0. 0	0. 1
Testis	94		1. 1	0. 6	4. 6	0. 6	0. 0	0. 1
Lymph node	93		1. 5	0. 8	4. 1	0. 4	0. 0	0. 1
Lung	91		1. 3	1. 0	3. 3	0. 1	0. 0	0. 0
Solid-ascites tumor	93	ascites	0. 9	0. 5	2. 0	0. 2	0. 0	0. 1
Ascites tumor	90		0. 9	0. 3	1. 0	1. 2	0. 1	0. 2
None	—	none	—	—	1. 9	0. 4	0. 1	0. 1
None	—	solid-ascites	2. 4	1. 4	3. 5	6. 4	0. 5	1. 0
Spleen	87	solid-ascites	1. 8	1. 2	6. 2	2. 1	0. 1	0. 4
Spleen	87	ascites	2. 0	1. 1	7. 3	0. 9	0. 0	0. 2
Liver	89	solid-ascites	1. 3	0. 8	4. 5	2. 5	0. 2	0. 6
Liver	89	ascites	1. 9	1. 1	5. 1	0. 5	0. 0	0. 2

*Ten mg. globulin from an anti-solid-ascites-lymphosarcoma antiserum (lot No. 391A) was absorbed with 100 mg. tissue sediment; the supernatant fluid was absorbed by 10 mg. tumor sediment; the eluate from the tumor sediment was assayed for localization.

†Net localization is the percentage of injected dose found in whole tissue minus the percentage of dose due to background, calculated as shown in table 1. The net localization ± 95 percent confidence limits of normal globulin is: tumor, 0.0 ± 1.6; liver, 0.0 ± 0.6; kidney, 0.0 ± 0.1; spleen, 0.0 ± 0.1. Values are averaged from the assay of 3 rats.

‡The assay tumor was the solid-ascites form of the Murphy lymphosarcoma.

remove a large portion of cross-localizing antibodies, enough still remains to be detected if concentrated by elution from a tumor preparation, such as the solid-ascites tumor, which contains a fair amount of normal-tissue components. In these purification procedures, then, the ascites tumor serves not only as a means of concentrating tumor-localizing antibodies, once it is fairly free from large amounts of cross-localizing material, but also as a further means of purification by selective absorption of tumor-localizing antibodies.

Effect of tissue-stroma components in tumor on production of antibody.—Since the ascites, solid-ascites, and solid tumors have, in that order, increasing amounts of tissue stroma, the effect of this stroma on the apparent antigen specificity of the tumor was studied with respect to antibody response. Globulins from 2 antisera prepared against each of the 3 lymphosarcoma forms (solid, solid-ascites, and ascites) were purified by absorption and elution from sediments of ascites and solid-ascites forms. The results are shown in table 5. It is apparent that the "localization titer" of tumor-localizing antibody rose as the purity of the tumor form increased and that the "cross-localization titer" fell at the same time. Moreover, the ascites-tumor sediment consistently concentrated tumor-localizing antibodies to a greater extent, and cross-localizing antibodies to a lesser extent, than the solid-ascites tumor sediment.

It should be pointed out that an antiserum, such as 16C, prepared against a tumor grossly contaminated with normal-tissue stroma, contained little or no tumor-localizing antibody although it still contained a fairly abundant amount of cross-localizing antibodies. Thus, the presence of cross-localizing antibodies in an antiserum to tumor does not necessarily mean that tumor-localizing antibodies are also present. On the other hand, an antiserum such as 513A, prepared against a much less contaminated tumor tissue, such as the ascites, contained a fairly high "localization titer" of tumor-localizing antibody and a low titer of cross-localizing antibodies. These contaminating antibodies, as shown in table 1, can be completely removed, for all practical purposes, by a single absorption with normal tissue.

Further concentration of localizing antibodies by double elution.—In order to concentrate tumor-localizing antibodies still further, we carried out 2 successive absorptions on and elutions from tumor sediments—termed double elution. In the first absorption-elution step, 10 mg. of radioiodinated globulin and 100 mg. of sediment were used; in the second, the eluate (containing about 2% of the initial radioactivity) was absorbed on 10 mg. of tumor sediment and a second eluate was subsequently obtained (containing about 0.1% of the initial radioactivity). Normal rabbit serum (0.2 ml.) was added to this eluate (10 ml.) before injection into rats.

When antibodies from an anti-solid-ascites-tumor antiserum were concentrated by double elution from solid-ascites tumor sediment, cross-localizing antibodies predominated; *e.g.*, 15 percent of the injected dose localized in liver (table 6). This concentration of cross-localizing antibodies was decreased by absorbing the globulin with spleen prior to double

TABLE 5.—In vivo *localization in tumor-bearing rats of antibodies prepared against original solid, solid-ascites, and free ascites forms of Murphy lymphosarcoma, which were concentrated by absorption on and elution from sediments of the solid-ascites and the free ascites forms*

Anti-tumor antiserum		*In vitro* absorption*			Net localization†			
Prepared against	Lot No.	Tumor sediment	Radioactivity		Tumor‡	Liver	Kidney	Spleen
			Absorbed (*percent*)	Eluted (*percent*)				
Solid-tumor sediment	16C	Solid-ascites tumor	2.0	0.8	0.0	5.3	0.4	1.0
		Ascites tumor	2.6	0.7	1.1	2.4	0.1	0.4
	32C	Solid-ascites tumor	2.4	0.6	2.1	4.9	0.4	0.9
		Ascites tumor	3.4	1.3	3.0	2.1	0.1	0.4
Solid-ascites-tumor sediment	386A	Solid-ascites tumor	1.8	0.7	2.9	4.9	0.3	0.8
		Ascites tumor	2.2	0.7	5.0	1.4	0.0	0.3
	389A	Solid-ascites tumor	2.1	0.9	3.8	6.9	0.6	1.0
		Ascites tumor	3.4	1.2	4.4	1.8	0.1	0.4
Free ascites tumor (whole)	512A	Solid-ascites tumor	4.5	1.6	5.8	1.3	0.0	0.4
		Ascites tumor	5.3	1.6	9.0	0.9	−0.1	0.2
	513A	Solid-ascites tumor	3.2	1.1	7.8	1.9	0.1	0.4
		Ascites tumor	4.5	1.3	9.3	1.1	0.0	0.3

*Ten mg. globulin was absorbed on and eluted from 10 mg. tumor sediment.

†Net localization is the percentage of injected dose found in whole tissue minus the percentage of dose due to background, calculated as shown in table 1. The net localization ±95 percent confidence limits of normal globulin is: tumor, 0.0±1.6; liver, 0.0±0.6; kidney, 0.0±0.1; spleen, 0.0±0.1. Values are averaged from assay of 3 rats.

‡The assay tumor was the solid-ascites form of the Murphy lymphosarcoma.

elution, or, still better, by using free ascites-tumor sediment for double elution. Indeed, the preparation with the highest tumor-localization titer (16%) and lowest cross-localization titer (3% in liver) was obtained by prior absorption with spleen and double elution from free ascites-tumor sediment.

TABLE 6.—In vivo *localization in tumor-bearing rats of antibodies concentrated by 2 successive absorptions on and elutions from tumor sediments (double elution)*

Antiserum globulin	Method of antibody concentration*		Net localization†			
	Sediment for prior absorption	Tumor sediment for double elution	Tumor‡	Liver	Kidney	Spleen
Anti-bovine-γ-globulin (pooled)	—	Solid-ascites	0. 1	−0. 2	0. 0	0. 0
Anti-solid-ascites-lymphosarcoma sediment (386A)	—	Solid-ascites	8. 7	15. 0	0. 6	3. 1
	Spleen	Solid-ascites	11. 0	7. 2	0. 2	2. 1
	—	Free-ascites	6. 2	2. 8	0. 0	0. 6
	Spleen	Free-ascites	16. 0	2. 6	0. 0	0. 5
Anti-free-ascites-lymphosarcoma, whole (513A)	—	Solid-ascites	17. 0	2. 4	0. 2	0. 6
	—	Free-ascites	12. 0	1. 6	0. 0	0. 5
	Spleen	Free-ascites	18. 0	−0. 2	0. 0	0. 0

*Antibodies from 10 mg. globulin (or from supernatant of 10 mg. absorbed with 100 mg. spleen sediment) were absorbed and eluted from 100 mg. tumor sediment; antibodies from this eluate were absorbed and eluted from 10 mg. tumor sediment; the second eluate (10 ml.), after addition of 0.2 ml. normal rabbit serum, was assayed in rats for localization activity.

†Net localization is the percentage of injected dose found in whole tissue minus the percentage of dose due to background, calculated as shown in table 1. The net localization ±95 percent confidence limits of normal globulin is: tumor, 0.0±1.6; liver, 0.0±0.6; kidney, 0.0±0.1; spleen, 0.0±0.1. Values are averaged from assay of 3 rats.

‡The assay tumor was the solid-ascites form of the Murphy lymphosarcoma in Wistar rats.

Similar results were obtained with an anti-free-ascites-tumor antiserum; however, since this serum contained only a relatively small amount of cross-localizing antibody to begin with, even the double elution from solid-ascites tumor sediment yielded a preparation with good tumor specificity. Prior absorption with spleen and double elution from ascites tumor resulted in a concentrated antibody solution with the highest tumor-localization titer yet obtained (18%) and with no detectable localization in liver, kidney, or spleen.

In contrast, no localizing antibodies were concentrated from a control antiserum, anti-bovine-γ-globulin, by this double elution process. It should be remembered, however, that the localization values here reported are net values above the background for nonlocalizing globulins. Hence, the total observed localization, including both net and background, for our most active preparation in this experiment actually was 20 to 25 percent of the total injected radioactive dose.

Summary

In the past, antisera which had been prepared against the Murphy rat lymphosarcoma, fractionated for globulin, and purified for antibodies by absorption and elution from lymphosarcoma sediment were found to cross-localize extensively in normal tissues.

In these experiments, similar antiserum globulins were absorbed with sediments of normal tissue to remove cross-localizing antibodies either prior or subsequent to absorption on and elution from tumor. Fractions so obtained had higher "localization titers" in tumor and much reduced "cross-localization titers" in normal tissues.

Cross-localizing antibodies were found to be due primarily to tissue stroma in the solid lymphosarcoma which had been used for antibody production and for absorption-elution procedures. When this stroma was eliminated by conversion of the tumor to an ascites form, and when the stroma-free ascites tumor was used for antibody production and for absorption-elution procedures, fractions of antiserum globulins were obtained with a high localization specificity for tumor.

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The Effect of Subcutaneous Inoculation of 4-*o*-Tolylazo-*o*-Toluidine in Strain HR Mice [1,2]

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The development of hemangioendotheliomas in a group of untreated strain HR mice, which carry the gene for hairless, has been reported previously (*1*). Tumors occurred in 33 of 178 animals at various sites, such as subcutaneous tissues, liver, spleen, ovaries, and mesentery. The frequent occurrence of spontaneous hemangioendotheliomas has not been reported in any other strains, although this type of tumor has been observed in strains A, C3H, C57BL, and BALB/c following the injection of 4-*o*-tolylazo-*o*-toluidine[5] (*2–6*). The results, just cited, prompted the present experiment in which a strain with a large number of spontaneous tumors of a particular type could be exposed to a carcinogen which had elicited the same tumor type in other strains. Groups of haired and hairless male and female mice of the HR strain were inoculated subcutaneously with 4-*o*-tolylazo-*o*-toluidine, and the effect of this compound on the incidence of hemangioendotheliomas in this strain was observed.

Materials and Methods

The strain HR mice were obtained in 1947 from Dr. F. G. Carnochan of Carworth Farms, whose stock had come from Dr. F. A. E. Crew in Edinburgh, Scotland. Since their arrival at the National Cancer Institute they have been inbred by brother × sister matings and are now in the 22nd generation of such inbreeding. Matings are made using haired (*Hr/hr*) females and hairless (*hr/hr*) males because the hairless females usually give insufficient milk to rear their young.

Four groups of mice, consisting of 36 haired males, 36 haired females, 29 hairless males, and 28 hairless females, were used as experimental animals. They were inoculated subcutaneously in the right axilla with 10 mg. of 4-*o*-tolylazo-*o*-toluidine[6] in 0.25 cc. of olive oil with a tuberculin syringe. The opening made by the needle was sealed with a drop

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[2] Portions of this material have been presented at the meeting of the American Association for Cancer Research, Atlantic City, N. J., April 13 to 15, 1956.
[3] The author gratefully acknowledges the technical assistance of Wayne D. Levillain.
[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[5] Formerly designated as o-aminoazotoluene.
[6] The compound was obtained from Dr. H. B. Andervont.

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of collodion to prevent seepage of the injected material (*6*). The animals were inoculated at 2 months of age and each month thereafter until they had received a total of 100 mg. To prepare the solution, the 4-*o*-tolylazo-*o*-toluidine was added to the olive oil and the 2 were heated gently and shaken. Fresh solutions were prepared for each monthly series of injections (*3*). Four groups, consisting of 25 haired males, 25 haired females, 34 hairless males, and 30 hairless females, were maintained as control animals.

The animals were kept in plastic cages, 8 to a cage, and were constantly supplied with food in pellets and tap water. They were examined at weekly intervals and, when tumors developed or the animals were moribund, autopsies were performed. Autopsies were not obtained on 54 mice (28 treated and 26 untreated animals) because the animals were found dead and the tissues autolyzed. Since the animals which died were approximately equally distributed between the treated and untreated groups, it can be assumed that the relative distribution of tumors was not altered materially. Tissues were fixed in Fekete's modification of Tellyesniczky's fixative (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part) and stained with hematoxylin and eosin.

Results and Discussion

The results are presented in table 1.[7] A significant increase in the incidence of hemangioendotheliomas was found in the treated animals as compared with the untreated animals ($P<0.001$). The untreated animals lived longer than the treated animals and for that reason the increase in incidence of hemangioendotheliomas among the latter could not be ascribed to increased survival. Fifteen of 23 haired and 12 of 22 hairless treated males developed hemangioendotheliomas as compared with 8 of 24 haired and 3 of 16 hairless untreated males. Twenty-two of 31 haired and 19 of 25 hairless treated females developed hemangioendotheliomas as compared with 6 of 23 haired and 6 of 25 hairless untreated females. There was no significant difference between the incidences of hemangioendotheliomas in haired and hairless treated males ($\chi^2 = 0.5335$, P is between 0.3 and 0.5) or in haired and hairless treated females ($\chi^2 = 0.1787$, P is between 0.5 and 0.7). This agrees with results obtained previously in strain HR mice (*1*), but is contrary to findings (*7*) in an experiment in which tumor development was studied in backcross mice which segregated for the hairless and pink-eye genes. In the latter case, a significantly greater number of hemangioendotheliomas occurred in the haired animals. The incidences of hemangioendotheliomas were higher in the haired and hairless treated females than in the haired and hairless treated males, but the differences were not significant (in the comparison of haired males with haired females, $\chi^2 = 0.2024$, P is between 0.5 and 0.7 and in that of hairless males with hairless females, $\chi^2 = 2.398$, P is between 0.1 and 0.2). No sex difference was observed in the occurrence

[7] The author is grateful to Dr. Thelma B. Dunn, Pathologist, of the National Cancer Institute, who confirmed the diagnosis of many of the lesions observed in the animals of this experiment.

TABLE 1.—*Incidence of hemangioendotheliomas and hepatomas in strain HR mice following subcutaneous injection of 4-o-tolylazo-o-toluidine*

Phenotype and sex	Group	Number of mice	Average age at death (*months*)	Hemangioendotheliomas			Hepatomas		
				Number with tumor*	Average age at death (*months*)	Tumor incidence (*percent*)	Number with tumor†	Average age at death (*months*)	Percentage of tumor
Haired ♂ ♂	Treated	23	17. 3	15(9)	18. 4	65. 2	9(4)	19. 3	39. 1
Hairless ♂ ♂	Treated	22	16. 4	12(2)	18. 3	54. 5	7(0)	18. 2	31. 8
Haired ♂ ♂	Untreated	24	22. 2	8(3)	23. 3	33. 3	5(0)	22. 6	20. 8
Hairless ♂ ♂	Untreated	16	17. 9	3(0)	18. 7	18. 8	1(0)	23. 0	6. 3
Haired ♀ ♀	Treated	31	17. 9	22(6)	18. 1	71. 0	25(18)	18. 8	80. 6
Hairless ♀ ♀	Treated	25	19. 9	19(8)	20. 2	76. 0	24(16)	20. 2	96. 0
Haired ♀ ♀	Untreated	23	23. 2	6(1)	23. 7	26. 1	1(0)	24. 0	4. 3
Hairless ♀ ♀	Untreated	25	21. 0	6(1)	22. 6	24. 0	0	—	0

*The figures in parentheses indicate the number of animals with multiple hemangioendotheliomas.
†The figures in parentheses indicate the number of animals with multiple hepatomas.

TABLE 2.—*Types of tumors found in treated and untreated strain HR mice in addition to hemangioendotheliomas and hepatomas*

Phenotype and sex	Group	Number of mice	Average age at death (*months*)	Type of tumor	Mice with tumors (*number*)	Average age at death (*months*)
Haired ♂♂	Treated	23	17. 3	Papilloma of skin	1	18. 0
				Fibrosarcoma—subcutaneous	2	19. 5
				Reticulum-cell neoplasm—Type A	2	16. 5
				Lymphocytic neoplasm	1	14. 0
				Pulmonary tumor	3	17. 7
Hairless ♂♂	Treated	22	16. 4	Papilloma of skin	4	15. 5
				Squamous-cell carcinoma of skin	2	14. 0
				Fibrosarcoma—subcutaneous	1	17. 0
				Pulmonary tumor	1	22. 0
				Tumor of vacuolated cells (lipoma?)	1	18. 0
Haired ♂♂	Untreated	24	22. 2	Papilloma of skin	1	25. 0
				Papilloma of pylorus	1	23. 0
				Squamous-cell carcinoma of lung	1	24. 0
				Reticulum-cell neoplasm—Type A	2	21. 0
				Reticulum-cell neoplasm—Type B	1	20. 0
				Pulmonary tumor	3	24. 0
Hairless ♂♂	Untreated	16	17. 9	Papilloma of skin	2	18. 5
				Reticulum-cell neoplasm—Type B	1	23. 0
Haired ♀♀	Treated	31	17. 9	Papilloma of skin	1	20. 0
				Reticulum-cell neoplasm—Type A	2	18. 5
				Lymphocytic neoplasm	1	9. 0
				Pulmonary tumor	3	20. 3
				Granulosa-cell tumor of ovary	5	19. 8
				Harderian-gland tumor	1	19. 0
Hairless ♀♀	Treated	25	19. 9	Papilloma of skin	4	22. 2
				Squamous-cell carcinoma of skin	2	20. 5
				Reticulum-cell neoplasm—Type A	4	19. 2
				Pulmonary tumor	5	21. 4

Haired ♀♀	Untreated	23	23.2	Fibrosarcoma—subcutaneous	1	23.0
				Fibrosarcoma—near kidney	1	23.0
				Reticulum-cell neoplasm—Type A	2	20.5
				Reticulum-cell neoplasm—Type B	3	25.7
				Lymphocytic neoplasm	1	23.0
				Pulmonary tumor	4	24.5
				Mammary gland tumor	2	25.0
				Papillary cystic tumor of ovary	1	26.0
				Granulosa-cell tumor of ovary	2	23.5
Hairless ♀♀	Untreated	25	21.0	Papilloma of skin	2	22.5
				Squamous-cell carcinoma of skin	3	18.0
				Carcinoma (from conjunctiva?)	1	22.0
				Fibrosarcoma of vagina	3	19.0
				Reticulum-cell neoplasm—Type A	3	23.3
				Pulmonary tumor	2	23.5

of hemangioendotheliomas in untreated strain HR mice in a previous experiment (*1*). Andervont *et al.* (*2,6*) reported that strain BALB/c females were more susceptible than males to the production of hemangioendotheliomas following subcutaneous inoculation of 4-*o*-tolylazo-*o*-toluidine. A higher percentage of hemangioendotheliomas was induced in strain HR mice in the present experiment than was induced previously in strains A, C3H, C57BL, and BALB/c, with the exception of the BALB/c females, in which the incidence was higher (*2*).

In a number of instances more than 1 hemangioendothelioma occurred in an affected animal (table 1). The same situation was observed by Andervont *et al.* (*2*). In the present experiment multiple tumors were observed more often in treated animals.

In addition to the increase in incidence of hemangioendotheliomas already mentioned, an increase in the incidence of hepatomas has been observed in strain HR mice following the subcutaneous inoculation of 4-*o*-tolylazo-*o*-toluidine (table 1). The susceptibility of various inbred strains to the induction of hepatic changes and of hepatomas has been demonstrated (*8,9*). Nine of 23 haired and 7 of 22 hairless treated males developed hepatomas as compared with 5 of 24 haired and 1 of 16 hairless untreated males. Twenty-five of 31 haired and 24 of 25 hairless treated females developed hepatomas as compared with 1 of 23 haired and none of 25 hairless untreated females. There was no significant difference between the incidences of hepatomas in the haired and hairless treated males ($\chi^2 = 0.2623$, P is between 0.5 and 0.7) or in the haired and hairless treated females ($\chi^2 = 2.983$, P is between 0.05 and 0.1). The incidence of hepatomas was high in the treated animals of both sexes but was significantly greater in females than in males (in the comparison of haired females with haired males, $\chi^2 = 9.758$, P is between 0.01 and 0.001, and in that of hairless females with hairless males, $\chi^2 = 21.468$, $P < 0.001$). This is in agreement with results obtained by Andervont *et al.* (*2–6*) in experiments employing strains A, C3H, C57BL, and BALB/c. Untreated males in the present experiment were more susceptible than females to the spontaneous development of hepatomas, as has been shown previously in the case of strain C3H mice (*10*). In another experiment (*1*), the relationship was of borderline significance in a group of untreated strain HR males and females.

Multiple hepatomas were observed in some of the treated animals but in none of the untreated animals (table 1).

Various other lesions were observed in the treated and untreated strain HR mice. These are listed in table 2. None of these occurred in significantly large numbers in either the treated or untreated animals. Similar lesions have been observed previously in untreated strain HR mice (*1*). Papillomas of skin, squamous-cell carcinomas of skin, pulmonary tumors, tumors of reticular tissue (lymphocytic neoplasms, reticulum-cell neoplasms, Types A and B) and subcutaneous fibrosarcomas occurred in a small number of animals. There were 3 fibrosarcomas of the vagina, 2 mammary gland tumors (1 adenocarcinoma Type A and 1 adenoacan-

thoma), 8 ovarian tumors, and 1 each of the following: Harderian-gland tumor, squamous-cell carcinoma in the lung (probably metastatic), carcinoma probably originating from the conjunctiva, and a tumor composed of vacuolated cells, possibly a lipoma.

Summary

Strain HR mice are susceptible to the development of spontaneous hemangioendotheliomas and hepatomas. An increase in the incidence of hemangioendotheliomas and of hepatomas has been observed in this strain of mice following the subcutaneous inoculation of 4-*o*-tolylazo-*o*-toluidine (*o*-aminoazotoluene). Hemangioendotheliomas induced with this compound occurred with greater frequency in strain HR mice than in mice of strains A, C3H, C57BL, and BALB/c, with the exception of the BALB/c females. In strain HR, females are more susceptible to the induction of hepatomas by 4-*o*-tolylazo-*o*-toluidine than are males.

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Errata for July 1956 Issue
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Erratum

Page 41: In the paper entitled "Quantitative Studies on the Multiplication of Neoplastic Cells *in Vivo*. III. Metabolic Stability of Deoxypentose Nucleic Acid and the Use of Labeled Tumor Cells for the Measurement of Growth Curves," by Révész, Forssberg, and Klein, in table 2, second column under "Inoculum," *for* "Dose" *read* "PNA:DNA."

Erratum

Page 65: In the paper entitled "Some Biochemical Differences in Normal and Tumor Tissue (Sarcoma 180) of Mice: Amino Acid Composition," by Mickelson and Barvick, fourth paragraph, first sentence, *for* "Sauberlich and Bauman analyzed samples of Flexner-Jobling carcinoma (a methylcholanthrene-induced sarcoma), two chemically induced hepatomas, . . . " *read* "Sauberlich and Bauman analyzed samples of Flexner-Jobling carcinoma, a methylcholanthrene-induced sarcoma, two chemically induced hepatomas, . . . "

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The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

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